# Elucidating the mode of action of gephyronic acid and related compounds inhibiting eukaryotic protein synthesis

Von der Fakultät für Lebenswissenschaften

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### Tagungsbeiträge

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# Contents

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# Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| Ala | Alanine |
| Asp | Aspartate |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| Caa*, C. albicans* | *Candida albicans* |
| CrPV | Cricket Paralysis Virus |
| DARTS | Drug Affinity Responsive Target Stability |
| DMEM | Dulbecco's modified Eagle's medium |
| E. coli | *Escherichia coli* |
| eEFs | Eukaryotic elongation factors |
| eIFs | Eukaryotic initiation factors |
| eRFs | Eukaryotic release factors |
| *et al.* | *et alii* |
| FACS | Fluorescence activated cell sorting |
| FBS | Fetal bovine serum |
| Fig | Figure |
| FITC | Fluorescein isothiocyanate |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GDP | Guanine diphosphate |
| Glu | Glutamate |
| GTP | Guanine triphosphate |
| HCV | Hepatitis C Virus |
| Hna, *H. anomala* | *Hansenula anomala* |
| IRES | Internal Ribosome Entry Sites |
| *K. pneumoniae* | *Klebsiella pneumoniae* |

| | |
|---|---|
| kDa | Kilo Dalton |
| m | Meter or milli |
| M | Molar |
| MEM | Minimum essential media |
| MIC | Minimum inhibition concentration |
| mRNA | Messenger RNA |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid |
| ng | Nanogram |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PAGE | Polyacrylamide gel electrophoresis |
| PC | Pyruvate complex |
| RNA | Ribonucleic acid |
| S | Svedberg |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SDS | Sodium dodecyl sulfate |
| Ser | Serine |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline-Tween |
| Thr | Theronine |
| tRNA | Transfer RNA |
| Tyr | Tyrosine |
| UTR | Untranslated region |
| WT | Wild type |

# Abstract

Protein biosynthesis is a vital, tightly regulated and a complex process involving a number of factors and enzymes. While the bacterial protein synthesis machinery is well understood due to the availability of various small molecule inhibitors, this is not true for the eukaryotic translation system. It is therefore of great interest to study small molecule translation inhibitors and elucidate their mode of action. This study involves four small molecules which target the eukaryotic translation system, i) gephyronic acid A, ii) des-epoxy tedanolide, iii) myriaporone 3/4, and iv) aetheramide B.

All these compounds inhibit growth of human cancer cell lines like KB-3-1 and A-431 in nanomolar ranges. Their cytotoxicity is attributed to the translation inhibition properties. *In vitro* and *in vivo* inhibition assays showed that the four substances inhibited translation with $IC_{50}$ values in the nanomolar range. Using bicistronic vectors the phase of translation inhibition was assessed. Gephyronic acid A and aetheramide B inhibited the initiation, myriaporone 3/4 the elongation. Des-epoxy tedanolide had targets in both phases. A Drug Affinity Responsive Target Stability (DARTS) approach was used to identify the targets of gephyronic acid and myriaporone 3/4. The results showed that gephyronic acid and myriaporone bind directly to the eukaryotic initiation factor 2 alpha (eIF2α) and the eEF2 (eukaryotic elongation factor 2) kinase, respectively. In both cases they are the first natural compounds identified to target these factors. *In silico* modelling studies suggested that the important epoxide group of gephyronic acid binds to Tyr 39 and Glu 15. The binding of gephyronic acid to eIF2α probably inhibits eIF2α interaction with the guanidine exchange factor eIF2B required for conversion of eIF2α-GDP to eIF2α-GTP. Using immunofluorescence techniques on treated cells it was shown that des-epoxy tedanolide inhibited translation by inducing the phosphorylation of eIF2α and eEF2. Aetheramide B inhibited translation via dephosphorylation of 4E-BP, i.e. by inhibiting the mTOR pathway.

# Zusammenfassung

Proteinbiosynthese ist ein zentraler, streng regulierter und komplexer Prozess, der eine Reihe von Faktoren und Enzymen einschließt. Während der bakterielle Proteinsynthese-Mechanismus gut verstanden ist, da verschiedene kleine Moleküle als Translationsinhibitoren zur Verfügung stehen, ist das für das eukaryontische Translationssystem nicht der Fall. Es ist deshalb von großem Interesse, niedermolekulare Translationsinhibitoren zu erforschen und ihre Wirkweise aufzuklären. Diese Studie befasst sich mit vier kleinen Molekülen, die mit dem eukaryontischen Translationssystem interagieren, Gephyronsäure A, Des-epoxy-Tedanolid, Myriaporon 3/4 und Aetheramid B.

Alle diese Verbindungen hemmen das Wachstum von humanen Krebszelllinien wie KB-3-1 und A-431 im nanomolaren Bereich. Ihre Zytotoxizität ist auf die Translations-hemmenden Eigenschaften zurückzuführen. Hemmtests *in vitro* und *in vivo* zeigten, dass die vier Substanzen die Translation mit $IC_{50}$-Werten hemmen, die im nanomolaren Bereich liegen. Die Phase der Translationshemmung konnte mit Hilfe bicistronischer Vektoren bestimmt werden. Danach hemmen Gephyronsäure A und Aetheramid B die Initiation, Myriaporon 3/4 die Elongation. Des-epoxy-Tedanolid hat Zielproteine in beiden Phasen. Mit Hilfe eines Drug Affinity Responsive Target Stability (DARTS) Ansatzes konnten die Zielproteine von Gephyronsäure und Myriaporon 3/4 identifiziert werden. Die Ergebnisse zeigten, dass Gephyronsäure und Myriaporon direkt am eukaryontischen Initiationsfaktor 2 alpha (eIF2α) bzw. an der eEF2(eukaryontischer Elongationsfaktor 2)-Kinase binden. In beiden Fällen sind es die ersten identifizierten Naturstoffe, die mit diesen Faktoren interagieren. Modellierungsstudien *in silico* legen nahe, dass die Bindungspartner der für die Wirkung wichtigen Epoxidgruppe der Gephyronsäure Tyr 39 and Glu 14 sind. Die Bindung von Gephyronsäure an eIF2α hemmt wahrscheinlich die Interaktion mit dem Guanidin-Austauschfaktor eIF2B, die für die Umwandlung von eIF2α-GDP zu eIF2α-GTP nötig ist. Mit Immunfluoreszenztechniken konnte an behandelten Zellen gezeigt werden, dass Des-epoxy-Tedanolid die Translation dadurch hemmt, dass es eine Phosphorylierung von eIF2α und eEF2 induziert. Aetheramid B wirkt über eine Dephosphorylierung von 4E-BP, d.h. über den mTOR-Signalweg.

# 1 Introduction

Protein synthesis is a vital, tightly regulated and a complex process involving a number of factors and enzymes. These factors and enzymes catalyse the assembly of the ribosome subunits, mRNA templates and tRNAs (Aitken and Lorsch, 2012; Clardy, 2006; Jackson et al., 2010; Sonenberg and Hinnebusch, 2009). The bacterial protein synthesis machinery is well elucidated due to the availability of various small molecule translation inhibitors of bacterial translation. These small molecule inhibitors have provided us with great insights into the structure of the ribosome and the various factors involved in the translation machinery. However, the scarcely available inhibitors of eukaryotic protein synthesis have limited our knowledge regarding the eukaryotic translation system (Poehlsgaard and Douthwaite, 2005; Schroeder et al., 2007; Tu et al., 2005). Also the bacterial and eukaryotic translation machineries are quite distinct making it difficult to extrapolate the understanding of the prokaryotic translation machinery to the eukaryotes. Only a few available inhibitors of prokaryotic protein synthesis inhibit eukaryotic translation which tells us that the two systems are quite different (Schroeder et al., 2007; Sonenberg and Hinnebusch, 2009). Results from a large number of studies suggest that translation is dysregulated in cancer and a few translation inhibitors have been tested in clinical trials for cancer therapeutics already showing promising results (Malina et al., 2011; Silvera et al., 2010). However, it is a surprise that little effort has been taken to find new inhibitors of eukaryotic translation. Although identifying new translation inhibitors and studying their mode of action is a daunting task, it is well worth the effort considering their therapeutic value and their role in better elucidating the translation system.

## 1.1 Overview of eukaryotic translation

Translation of mRNA into its protein counterpart takes place in four well regulated steps viz. initiation, elongation, termination and recycling of the ribosome (Fig. 1.1). Translation initiation is the most important and rate limiting step of translation. It involves the assembly of the ribosomal subunits at the AUG start codon with the aid of wide range of initiation factors. In eukaryotes, initiation can be cap-dependent or cap-independent. In elongation, the cognate charged aminoacyl tRNA binds to the respective codon at the acceptor (A) site. A peptide bond is formed between the amino acid in the A site and the amino acid at the peptidyl (P) site. Then the ribosome translocates to the exit (E) site leaving the A site open for the next aminoacyl tRNA. The process continues till a stop codon is reached leading to the termination of the

peptide formation. Finally, the ribosomal subunits are dissociated and recycled back to initiation phase (Kapp and Lorsch, 2004; Marintchev and Wagner, 2004).

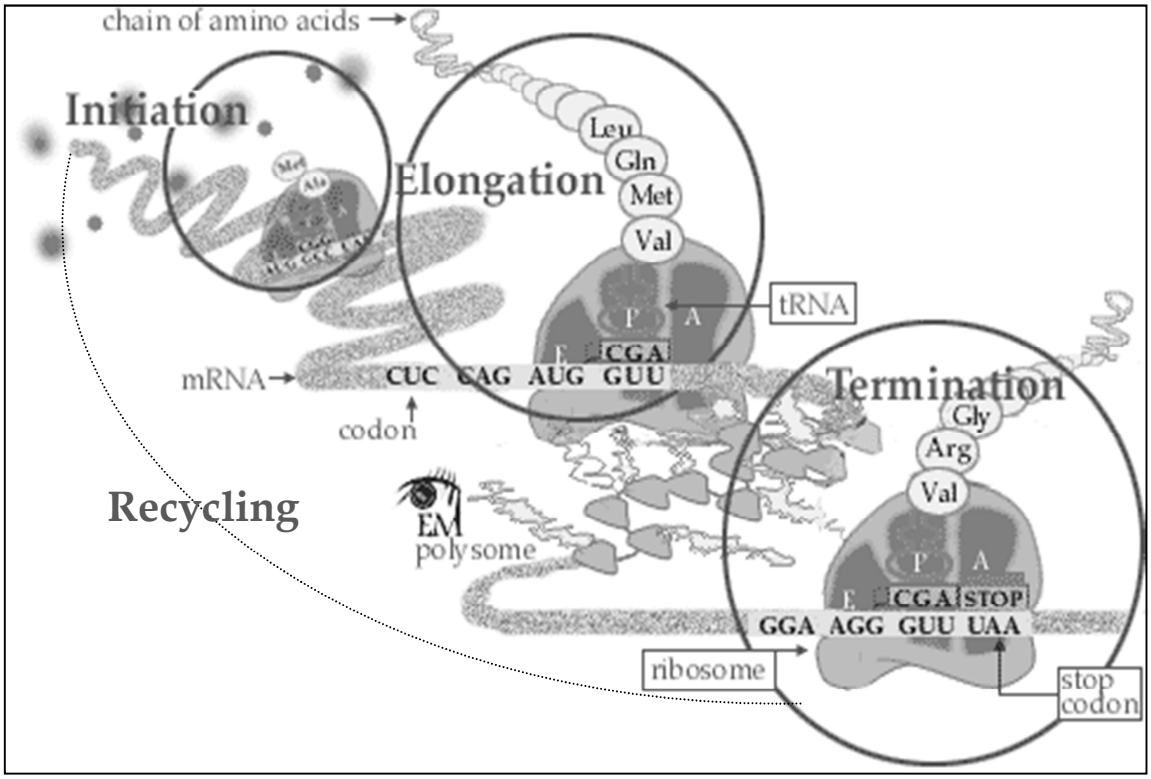



***Figure 1.1: Overview of translation in eukaryotes.***

*Translation is divided into initiation, elongation, termination and recycling of the ribosomal subunits (adapted from http://www.nobelprize.org/educational/medicine/dna/a/translation/).*

## 1.2 Translation initiation

The process of eukaryotic translation initiation involves the assembly of 40S and the 60S ribosome to form an elongation competent 80S ribosome along with initiator methionyl transfer RNA (Met-tRNA$_i$) at the P site on the start codon (AUG). This tightly regulated assembly takes place with the help of 12 eukaryotic initiator factors (eIFs) at least in the case of cap-dependent translation. The established mechanism of the initiation process can be divided into: i) formation of the ternary complex, ii) formation of the 43S preinitiation complex (PIC), iii) attachment of the 43S PIC to the mRNA, iv) scanning of the mRNA untranslated regions (UTRs) and identification of the start codon and v) joining the ribosomal subunits (Fig. 1.2) (Aitken and Lorsch, 2012; Jackson et al., 2010; Van Der Kelen et al., 2009). These stages of the initiation process are described in detail in the following sections.

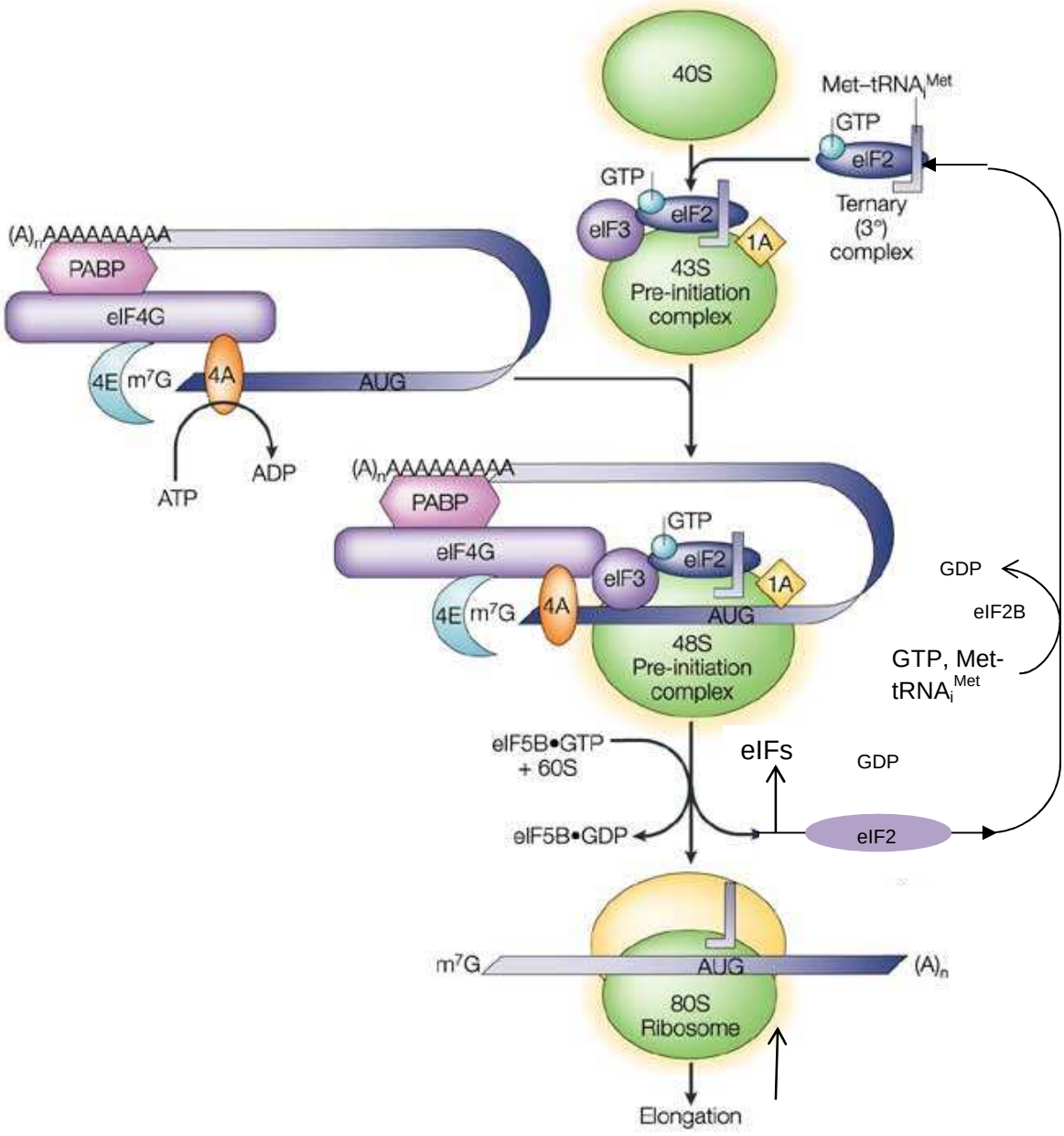



***Figure 1.2: Overview of translation in eukaryotes***.

*eIF2-GTP binds to the methionyl transfer RNA (fMet-tRNA$_i$) to form the ternary complex. The ternary complex then binds to the 40S ribosomal subunit to form the 43S complex. The eIF4F complex containing eIF4E, eIF4A and eIF4G binds to the 5'mRNA cap along with eIF4B. The eIF4 complex unwinds the secondary structures of the mRNA and recruits the mRNA to the 43S complex to form the 43S preinitiation complex (PIC). The PIC then scans the mRNA to locate the start AUG codon. Once the codon is reached eIF2-GTP undergoes hydrolysis and releases the tRNA$_i$. eIF5B helps in releasing of the initiation factor and the 60S subunit joins the 40S subunit to form the elongation competent initiation complex (modified from Klann and Dever, 2004).*

### 1.2.1 Formation of the ternary complex

The Met-tRNA$_i$ (first amino acid) is brought in by eIF2. eIF2 is a heteromer consisting of the α, β and γ subunits with a total molecular mass of 126 kDa. The γ subunit of the eIF2 complex is homologous to the eEF1A (Kapp and Lorsch, 2004; Naranda et al., 1995). Unlike eEF1A, which binds to all the tRNAs with the same affinity, eIF2 binds specifically to Met-tRNA$_i$ through the γ subunit. The γ subunit interacts directly with the GTP and the tRNA as shown by footprinting data. The specificity arises due to subtle differences which include three consecutive G:C pairing in the anti-codon stem, conserved sequences around the T loop, base pairing of A1:U72 at the acceptor stem, and modification of the phosphoribosyl unit at position 64 (Astrom et al., 1993). Of these the acceptor stem modification seems to be the most important for differentiating a tRNA into an initiator tRNA whereas the change at position 64 reduces the affinity of eEF1A for initiator tRNA (Fig. 1.3) (Drabkin et al., 1998). Met-tRNA$_i$ has a higher affinity for eIF2-GTP than for eIF2-GDP. The conversion of eIF2-GDP to eIF2-GTP takes place with the help of the guanine exchange factor eIF2B. This conversion is a well regulated stage in translation (Sonenberg and Hinnebusch, 2009). eIF2B, a heteropentamer consists of α, β, γ, δ, and ε subunits with a molecular mass of ~263 kDa. The catalytic subunits are δ and ε, which are responsible for guanine exchange. The other subunits form the regulatory unit of eIF2B (Stolboushkina and Garber, 2011).

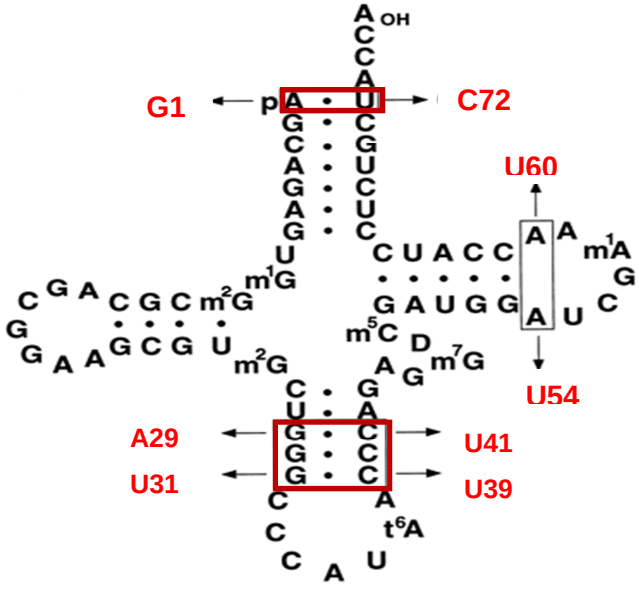



***Figure 1.3: Structure of eukaryotic tRNA.***

*The boxes indicate unique features of the initiator tRNA and the arrows the mutation in the tRNA to differentiate the initiator tRNA from the elongation tRNAs (adapted from Drabkin et al, 1997).*

### 1.2.2 Formation of the 43S preinitiation complex

The next in the initiation process is the formation of a 43S preinitiation complex (PIC). The ribosome required for the complex is acquired from recycling of the post termination complex. Formation of 43S preinitiation complex requires a pool of detached 40S and 60S ribosome subunits. Physiological conditions in the cell favour the association of the 40S and the 60S ribosomal subunits to form the 80S ribosome. Therefore it is necessary to dissociate the subunits (Jackson et al., 2010). The intact 80S ribosome from the post termination complex (TC) is still bound to mRNA, tRNA and the eukaryotic release factor 1 (eRF1) (Pisarev et al., 2007). The dissociation of the 80S ribosome into the 40S and 60S subunits and the removal of the bound mRNA and eRF1 takes place with the help of eIF3, eIF1 and eIF1A (Preiss and M, 2003). The binding of eIF3j induces the release of the tRNA from the post TC. eIF3, eIF1 and eIF1A remain bound to the 40S subunit to prevent re-attachment of the subunits. eIF3, eIF1 and eIF1A along with eIF5 promote the attachment of the ternary complex to the 40S ribosomal subunit forming the 43S preinitiation complex. Studies have shown that eIF3 binds near the P site and eIF1A binds near the A site of the 43S complex. The ternary complex is positioned at the P site. As eIF3 is a multimeric protein, it is equivalent to the 40S subunit in size and interacts with almost every component in the preinitiation complex (Fig. 1.4). It has been hypothesised that eIF3, eIF1A and eIF2 interact with each other to stabilise the ternary complex (Asano et al., 2000; Olsen et al., 2003; Phan et al., 1998).

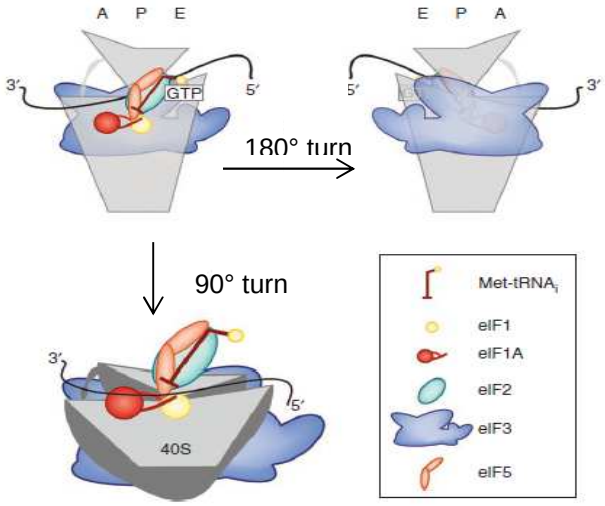



***Figure 1.4: Formation of PIC.***

*The two figures on the top show the positioning of the tRNA$_i$ and the initiation factors and the bottom panel shows the interactions of the initiation factors with the 40S subunit (adapted from Aitken and Lorsch, 2012).*

### 1.2.3 Attachment of the 43S PIC to the mRNA

The 43S PIC formation is followed by the recruitment of the mRNA to the complex. The 5' untranslated regions (UTRs) of the mRNA possess many secondary structures which hinder the loading of the 43S preinitiation complex onto the mRNA. The unwinding of the secondary structures and recognition of the 5' UTRs of the mRNA takes places with the help of the eIF4F, eIF4B and poly (A) binding protein (PABP) (Fig. 1.5) (Pestova and Kolupaeva, 2002). eIF4F is a multimeric protein composed of eIF4E, eIF4A and eIF4G. eIF4E, the smallest part of the eIF4F complex is a monomer of 25 kDa. eIF4E specifically recognises the 5' end and binds to the $m^7$GpppG5' cap structure of the UTR mRNA (Marcotrigiano et al., 1999). eIF4A is a monomeric protein with a molecular mass of 46 kDa. It belongs to the family of DEAD box RNA helicases and unwinds the secondary structures in the UTRs of the mRNA. Like other helicases eIF4A it is an RNA dependant ATPase and therefore uses the energy from ATP to unwind the RNA. On its own, eIF4A is a week ATPase and requires the help of eIF4B and eIF4G to enhance its ATPase activity (Bi and Goss, 2000; Rogers et al., 2002).

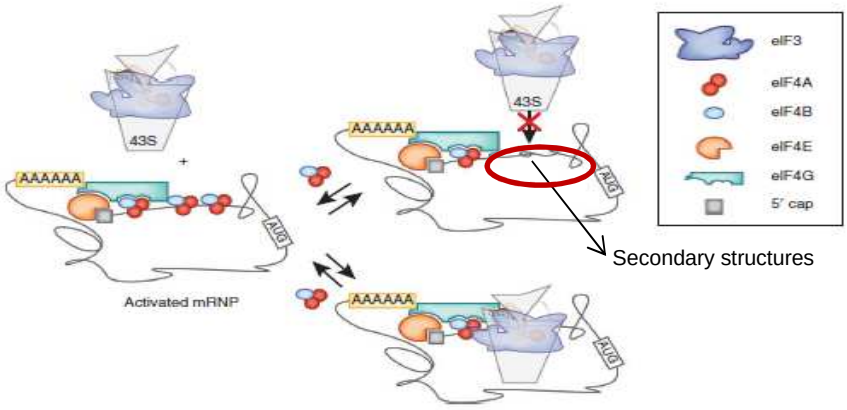



***Figure 1.5: Loading of the PIC***

*The mRNA has many secondary structures and as a result the 43S complex cannot bind to it. However, eIF4A along with eIF4B unwinds the secondary structures in the mRNA attached to eIF4E and eIF4G, and forms the activated mRNP. The activated mRNP then binds to the 43S complex to form the 43S PIC. The eIF3 bound to the 43S complex interacts with eIF4G and stabilises the PIC (adapted from Aitken and Lorsch, 2012).*

eIF4G is the largest part and the centrepiece of the eIF4F complex as it binds to eIF4E, eIF4A, 43S complex, PABP, eIF3 and the mRNA. It forms a bridge between the 5' cap of the mRNA and the 40S subunit. The eIF4G – eIF3 interaction brings the 43S complex and the mRNA together (De Gregorio et al., 1999; Morino et al., 2000) (Fig. 1.5).

### 1.2.4 Scanning of the mRNA UTRs and identification of the start codon

Once the 43S complex has attached to the 5' cap it begins to scan the mRNA downstream of the cap. The term scanning was coined by Kozak (Kozak, 1999). Scanning involves unwinding the secondary structures at the 5' cap and movement of the ribosome along the mRNA from the 5' end to the 3' end. It has been shown that the 43S complex can move along the unstructured mRNA without the help of the initiation factors. However, the complex does not have the ability to reach the AUG codon without the help of eIF1 and eIF1A (Passmore et al., 2007; Pestova and Kolupaeva, 2002). This suggests that the conformation induced by eIF1 and eIF1A is necessary for the scanning process. Experiments have also shown that the scanning is an energy consuming process. The energy is supplied in the form of ATP. Apart from ATP the scanning process also requires eIF4G, eIF4A and eIF4B. It is clear that eIF4F complex is not only required for the recruitment of the mRNA but also for scanning it (Jackson, 1991; Svitkin et al., 2001).

Although the molecular mechanism of scanning is still unclear two mechanisms have been proposed in an attempt to explain the actual process. According to the Brownian ratchet model, the eIF4 complex is bound to the E site of the 43S complex, *i.e.*, the trailing end. This prevents the ribosome from sliding backwards. As a result eIF4A acts as the helicase and the 40S subunit unwinds the mRNA by itself. This model is supported from the cryoelectron microscopy images which show the eIF4G bound downstream of the scanning direction, at the E site (Siridechadilok et al., 2005; Spirin, 2009). In the second model, it has been proposed that the eIF4A and eIF4B are present ahead of the PIC and unwinds the secondary structures. Backsliding is prevented by the formation of the secondary structures in the downstream region just behind the E site. In either of the models, the 40S subunit movement is assumed to proceed via diffusion (Marintchev et al., 2009; Pisarev et al., 2008). However, what actually might be going on during scanning still requires more study probably with small molecules, electron microscopy, etc. (Aitken and Lorsch, 2012).

Once the PIC starts scanning it is now competent to recognise the start codon. The actual model of start codon recognition was proposed by Kozak (Kozak, 1999). According to his model, the PIC identifies the first AUG closest to the 5' end. To ensure the fidelity of start codon identification the consensus sequence GCC(A/G)CC**AUG**G aids the whole process. The fidelity of the start codon recognition is maintained by eIF1. eIF1 ensures that the PIC differentiates between the correct AUG codon (marked by the consensus sequence) and the false AUG codons. A wide

range of studies have shown that eIF1 plays an acute role in start codon selection (Kozak, 1991; Pestova et al., 1998). Various proposed models explain the codon selection and base-paring, however, the Pestova and Kolupaeva model has wide acknowledgement. According to this model, the 43S complex in association with the eIF1 and eIF1A forms the 'open' complex. This complex favours the scanning process (Pestova and Kolupaeva, 2002). To form a stable codon anti-codon base pairing, the 'closed' conformation of the ribosomal complex has to be attained. This is accomplished by tightening of the eIF1A and 40S subunit bonding along with the detachment of the eIF1 from the P site. In the 'closed' state the anticodon tRNA is in the predisposed state to form the base pairing with the AUG codon on the mRNA. Also, this induces the Pi release from the eIF2 complex, converting the eIF2-GTP to eIF2-GDP. The hydrolysis of GTP to GDP takes place with the help of eIF5, a GTP activating protein dedicated to eIF2. The GTP hydrolysis leads to a reduction in the affinity of eIF2 for the Met-tRNAi. As a result eIF2 is partially displaced from the 40S ribosomal subunit and the already predisposed tRNAi base pairs with the start codon (Kapp and Lorsch, 2004; Maag et al., 2006; Maag et al., 2005).

### 1.2.5 Joining the ribosomal subunits

The final step in the translation initiation is the joining of the 40S and 60S ribosomal subunits. However, the joining of the ribosomal subunits can take place only after all the initiation factors have dissociated from the mRNA-ribosome complex. The dissociation of the initiation factors eIF1, eIF1A, eIF2 and eIF3 are promoted by the GTP hydrolysing eIF5B (Pestova et al., 2000). Once the tRNAi is base paired with the right codon, 60S subunit joining to the 40S subunit is automatically induced to form the 80S ribosome. The subunit joining prompts the eIF5B to hydrolyse GTP inducing a conformational change in the 80S ribosome. The GDP-eIF5B then freely dissociates from the 80S ribosome. After the release of the bound factors, the 80S ribosome along with the tRNAi and the mRNA form the 80S initiation complex which is ready for the elongation phase of protein synthesis (Acker et al., 2009; Kapp and Lorsch, 2004; Poyry et al., 2007).

### 1.2.6 Cap-independent translation initiation

About 20 years ago, it was discovered that mRNA from picornavirus was translated quite differently from the cap-dependent translation. The ribosome was loaded onto a highly structured 5' cap region of the mRNA which initiated translation then. These highly structured regions of the viral mRNA were named Internal Ribosome Entry

Sites (IRES) (Balvay et al., 2009). The experimental evidence that picornavirus could translate in a cap-independent fashion was provided by tests with encephalomyocarditis virus (EMCV) and poliovirus (Jang et al., 1988; Nomoto et al., 1976). In these studies the 5' UTRs from these viruses were placed into a bicistronic vector where one of the cistrons had 5' UTRs from the cap-dependent translation system. The translation of the cap-dependent UTR was inhibited whereas the 5' viral UTR translation proceeded without any hindrance. Since then it has become clear that translation can also proceed in a cap-independent fashion.

Initially it was believed that the IRES elements could arise only from the viral mRNA but later studies led to the discovery of IRES elements even from the cellular mRNA. The first cellular IRES identified was that of BiP, an immunoglobulin heavy chain binding protein. The proteins translated via cellular IRES include a few translation initiation factors, survival proteins, oncogenes, etc. As of now 80 cellular IRES and 56 viral IRES have been studied (Baird et al., 2006; Mokrejs et al., 2006; Sarnow, 1989). Depending on the dispensability of the initiation factors and the secondary structures of the 5' UTR the viral IRES can be divided into Type 1 and 2 belonging to the picornavirus family, Type 3 of the flavivirus family and Type 4 of the dicistroviral family (Jackson et al., 2010).

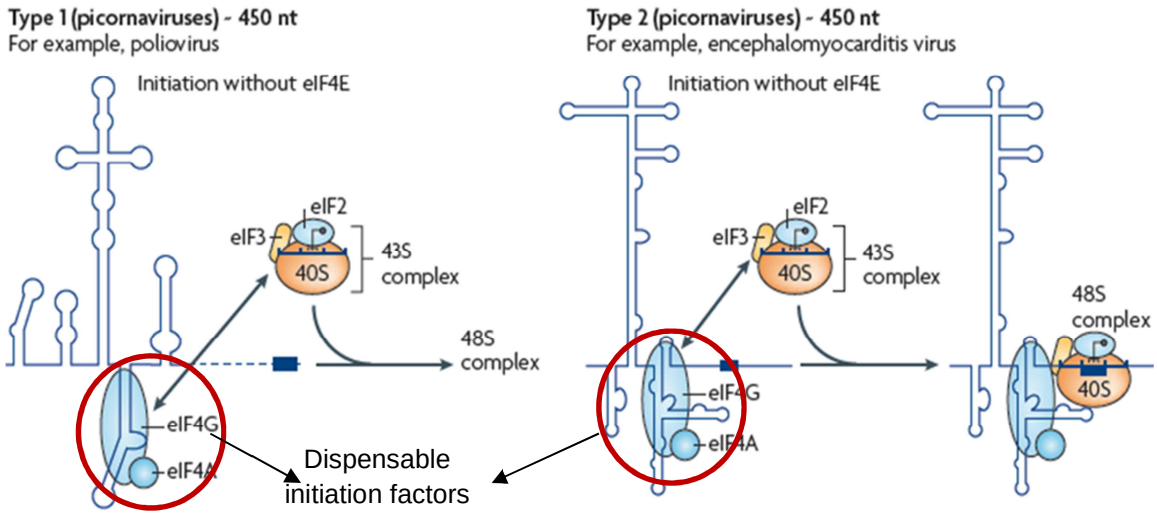



***Figure 1.6: Type 1 and type 2 IRES***

*Type 1 and type 2 IRES belong to the picornoviral family. Both the IRES do not require eIF4E. The 43S complex is only recruited with the help of eIF4A and eIF4G (adapted from Jackson et a.,l 2010).*

From the experimental data the picornoviral IRES was divided into type 1 as found in poliovirus and type 2 as in EMCV. Type 1 IRES elements use the second AUG codon 100 to 150 nucleotides downstream of a polypyrimidine region in the vicinity of the first codon whereas in the type 2 IRES the ribosome is loaded directly on the AUG codon 20 to 25 nucleotides downstream of the pyrimidine rich region. Experiments using reconstituted rabbit reticulocytes lysates identified that eIF4E and eIF4G were dispensable to the type 1 and 2 IRES elements, whereas all the other initiation factors were absolutely necessary for the translation (Balvay et al., 2009; Jang, 2006).

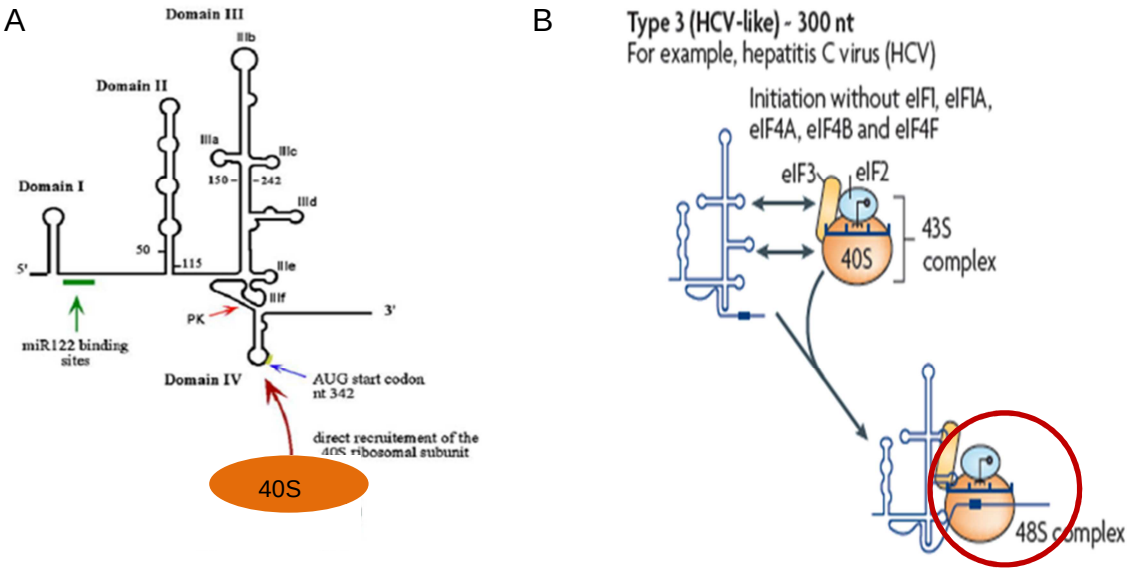



***Figure 1.7: HCV IRES***

*Panel A shows the secondary structures of type 3 IRES from HCV (adapted from Balvay et al., 2009). Panel B shows the HCV's requirement for the initiation factors for translation initiation (adapted from Jackson et a.,l 2010.)*

Members of flaviviral family include hepatitis C virus (HCV), bovine viral diarrhoea virus (BVDV), etc. The well characterised IRES of this family is that of HCV (Wang et al., 1993). It has a 341 nucleotide long 5' UTR and lacks the cap structure. As can be seen from Fig. 1.7A, these 341 nucleotides are highly structured into four domains. The start codon AUG is at position 342. The domains II, III and IV are essential for the IRES activity. In fact, the domain IV extends into the start codon. Once again with the use of rabbit reticulocyte lysates it was shown that type 2 does not require eIF4A, eIF4b and eIF4F for the ribosome loading. The ternary complex recruited via eIF3's interaction with domain IV and the IRES interacts directly with the 40S subunit. The IRES also plays a role in positioning the start codon in the P site by its direct

interaction with the 43S complex. Then eIF5B hydrolyses eIF2-GTP to eIF2-GDP thus releasing the $tRNA_i$ and allowing the joining of the 60S and the 40S subunits to form a translation elongation competent 80S initiation complex (Reynolds et al., 1996; Reynolds et al., 1995).

The last of the IRES types, type 4, belongs to the dicistrovirus. The examples of this class of IRES are cricket paralysis virus (CrPV), Drosophila C virus (DCV), etc. The dicistrovirus has two ORFs (open reading frames) separated by an intergenic region (IGR). The IGR region is approximately 200 nucleotides in length. The IRES following the IGR is capable of initiating translation at CAA codon using glutamine as the initiator amino acid. The IGR has three pseudoknot (PK) domain structures: PKI, PKII, and PKIII (Fig. 1.8) (Balvay et al., 2009; Reavy and Moore, 1983; Wilson et al., 2000). Crystallographic studies revealed that the CrPV IGR IRES includes the $tRNA_i$ binding E and P site. The CrPV IRES is quite distinct from the other IRES types in that it requires none of the initiation factors, the Met-$tRNA_i$ or energy in the form of GTP hydrolysis to initiate the translation process (Costantino et al., 2008; Pestova et al., 2004) (See Fig. 1.8, panel B). The non-requirement of Met-tRNAi is due to the presence of PKIII. PKIII is capable of binding to the P site of the 40S ribosome. The positioning of the PKIII on the P site resembles the tRNAi-mRNA conformation and enables recruitment of the 60S ribosome. The cytoplasmic environment is suitable for the joining of the two subunits. Hence, the 40S and 60S subunits join without any external stimulation and form an elongation competent 80S initiation complex (Balvay et al., 2009; Kanamori and Nakashima, 2001).

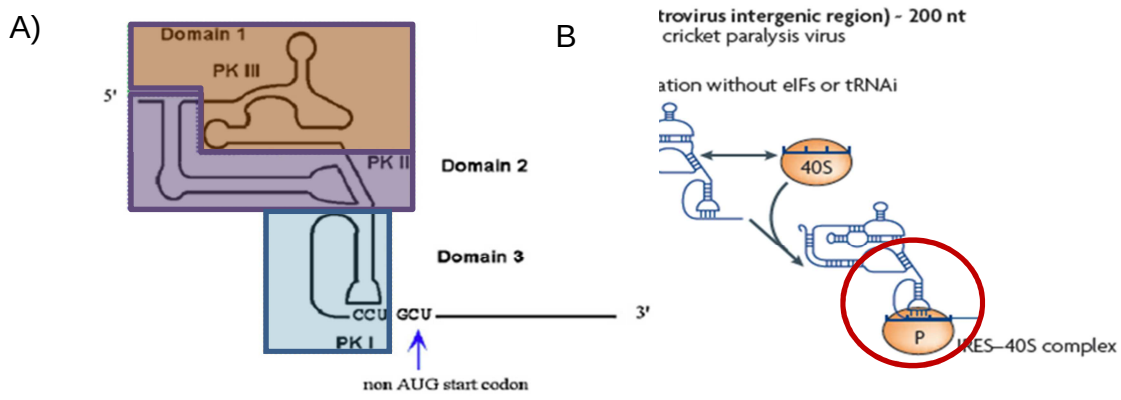



***Figure 1.8: CrPV IRES***

*Panel A shows the secondary structures of the type 4 IRES and the interaction between the pseudoknot domains (adapted from Balvay et al., 2009). Panel B shows the interaction of the 40S subunit with the type 4 IRES (adapted from Jackson et al., 2010).*

### 1.2.7 Regulation of translation initiation

Regulation of translation is vital for the cellular processes. Dysregulated translation can lead to various cellular disparities like cancer. In fact, it has been shown that in most cancers a number of initiation factors have been either up-regulated or down-regulated (Malina et al., 2011). Hence control of protein synthesis is very important. Of all the steps in translation, initiation is the rate limiting step which has many layers of regulation. The regulation can either be directly on the initiation factors or at the mRNA level (Jackson et al., 2010; Kapp and Lorsch, 2004).

Regulation of translation at the mRNA level takes place by PABP, RNA-protein interaction at the cap, by micro RNAs, regulation of 3'UTR, and interaction with proteins. The factors involved in the regulation are eIF2 and eIF4E which are parts of the eIF4F complex (Jackson et al., 2010).

Initiation factor eIF2 is regulated by phosphorylation at Ser 51 of the alpha subunit. As mentioned in Section 1.2.1, eIF2 is only active when bound to GTP. If GTP is hydrolysed to GDP then eIF2-GDP has to be converted back to the eIF2-GTP form. This conversion takes place with the help of the guanine exchange factor eIF2B. eIF2B has higher affinity for the phosphorylated eIF2 than to the non-phosphorylated protein. Hence, eIF2B is not available for guanine exchange (Vattem and Wek, 2004; Zhou et al., 2008) (Fig. 1.9).

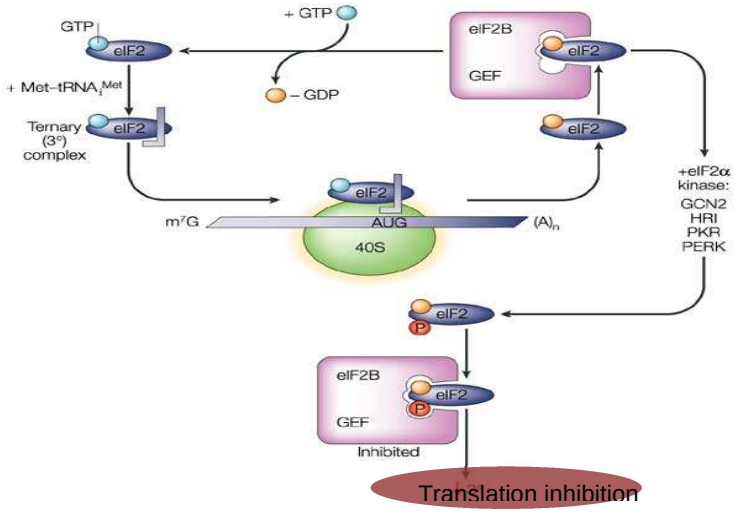



***Figure 1.9: Regulation of eIF2α.***

*eIF2-GTP associates with the Met-tRNAi to form the ternary complex and binds to the 40S. At a later stage of initiation, the GTP hydrolyses to GDP and the eIF2-GDP is released. The eIF2-GDP has to be converted back to eIF2-GTP to be active with the help of eIF2B (modified from Klann and Dever, 2004).*

eIF2 can be phosphorylated by four different kinases, viz. PKR (protein kinase R) which is activated by the presence of double stranded RNA, GCN2 (general control nonrepresed) which is activated by amino acid starvation, or UV radiation, HRI (heme regulated inhibitor) which is activated during hypoxia and only significant in red blood cells, and finally PERK, the PKR like endoplasmic reticulum kinase which is activated during ER stress. eIF2 phosphorylation can be induced by heat shock, UV rays, viral infection, nutrient deprivation, etc. (Klann and Dever, 2004).

The second initiation factor to be regulated is eIF4E. As it is a comparatively low available factor among the initiation factors, it is thought to be the major rate limiting component of translation initiation. eIF4E can be regulated at two levels. Firstly it can be regulated by direct phosphorylation and secondly by altering the phosphorylation state of the eIF4E binding protein, 4E-BP. eIF4E can be phosphorylated by MAP kinase interacting Ser/Thr kinase 1 and 2 (MNK1 and MNK2) at Ser 209. This phosphorylation takes only place when eIF4E is bound to eIF4G. The second mechanism of eIF4E regulation involves its availability to form the eIF4F complex. When eIF4E is bound to 4E-BP it is unavailable for binding to eIF4G. If 4E-BP is phosphorylated it's affinity for eIF4E is reduced (Fig. 1.10). 4E-BP can only bind to eIF4E when it is hypo-phosphorylated. Thus, when eIF4E is not bound to 4E-BP it is free to interact with eIF4G and proceed with the translation. The phosphorylation state of 4E-BP can be altered by various stimuli like insulin, adenoviral infection, interleukins, etc. (Gingras et al., 1999; Jackson et al., 2010).

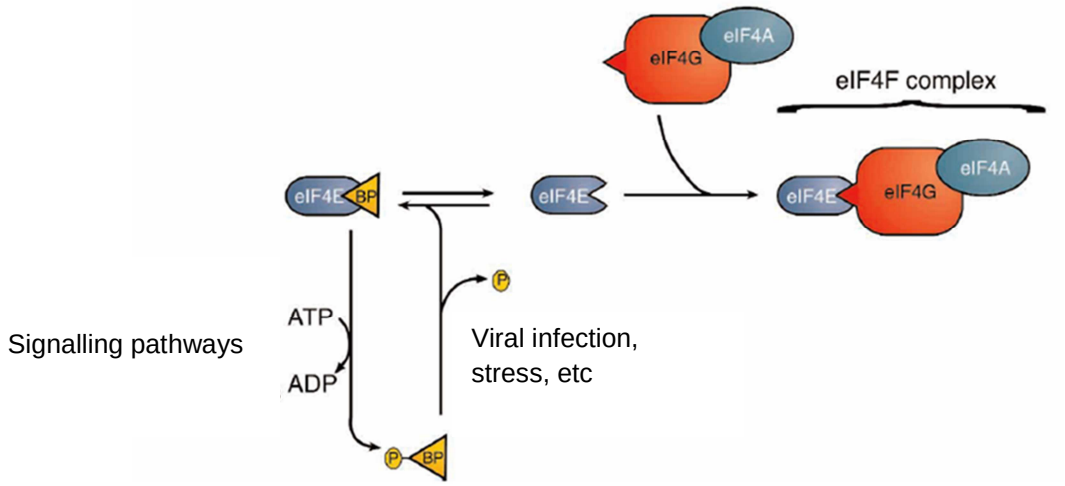



***Figure 1.10: Regulation of eIF4E by phosphorylation of 4E-BP***

*Both eIF4G and 4E-BP compete with each other to bind to eIF4E. However, if 4E-BP is hyper-phosphorylated it has low affinity for eIF4E and thus eIF4E is freely available to interact with eIF4G to form the eIF4F complex (Gingars et al., 1999).*

The mTOR signalling pathway can also influence the rate of translation initiation. As seen from Fig. 1.11, mTOR also alters the phosphorylation state of 4E-BP. mTOR phosphorylates the threonine residues at position 37 and 46 of 4E-BP. This phosphorylation is essential for phosphorylation at the C terminal end which is required for its inactivation. mTOR also phosphorylates ribosomal 40S protein S6 kinase (S6K). The activated kinase phosphorylates S6 which in turn regulates mRNAs coding for ribosomal protein. The activated S6 can also interact with eIF3 and eIF4B. Thus, translation can be inhibited by inactivating mTOR as in the case of rapamycin (Van Der Kelen et al., 2009).

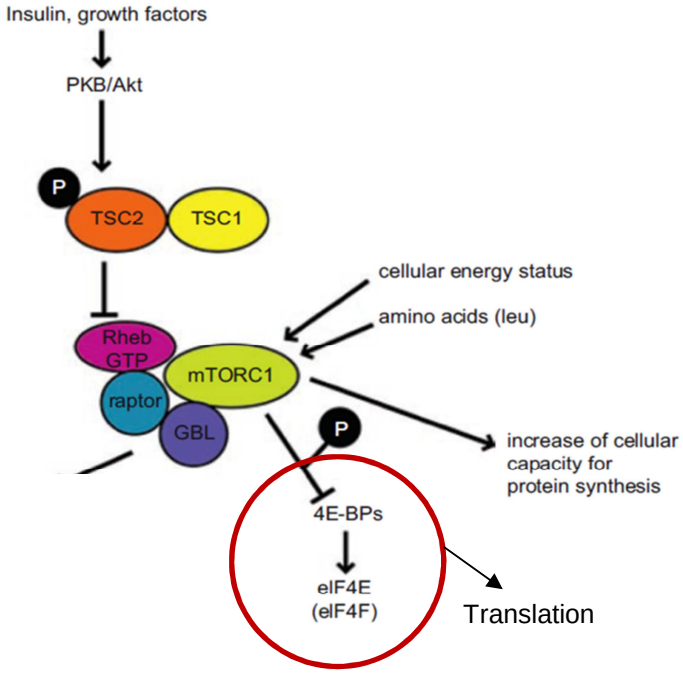



***Figure 1.11: Regulation of translation by mTOR pathway***

*When mTOR is activated it induces phosphorylation of 4E-BP. Thus the hyper-phosphorylated 4E-BP is dissociated from eIF4E making eIF4E freely available for forming the eIF4F complex (adapted from Kelen et al., 2009.)*

## 1.3 Translation elongation

Unlike initiation, elongation is highly conserved between eukaryotes and prokaryotes. Much of our understanding of the eukaryotic elongation process comes from the bacterial machinery (Kapp and Lorsch, 2004; Rodnina and Wintermeyer, 2009). Elongation proceeds once the 80S elongation competent initiation complex is formed. In the 80S elongation competent complex, the initiator Met-tRNA is bound to the start AUG codon and is positioned on the P site, leaving the A site free for the entry of next

amino acyl tRNA (aa-tRNA). eEF1A-GTP brings in the next aa-tRNA to the empty A site. The eEF1A-GTP-amino acyl tRNA form the ternary complex of the elongation phase. eEF1A is equivalent to EF-Tu in bacteria (Dever and Green, 2012; Ogle et al., 2001). Though any elongation ternary complex can enter the A site, fidelity is maintained by conformational changes in the anti-codon stem of the tRNA and the codon of the mRNA. The fidelity is also maintained by the hydrolysis of GTP which takes place only when the right aa-tRNA binds to the cognate codon (Schmeing et al., 2011). Base pairing of the cognate aa-tRNA with the codon of the mRNA brings about a conformational change in three nucleotide bases in the rRNA induced. As a result the bases interact with the mRNA-aa-tRNA complex. This interaction initiates the eEF1A's GTPase activity leading to hydrolysis of GTP to GDP and the release of aa-tRNA from eEF1A-GDP to the A site. The eEF1A-GDP is recycled to eEF1A-GTP with the help of guanine exchange factor eEF1B (Dever and Green, 2012; Voorhees et al., 2010).

The peptide bond between the amino acid at the P site and the A site is catalysed by ribosomal peptidyl transferases. The deacylated tRNA remains in a P/E hybrid state. In this state the acceptor end is bound to the E site and the anticodon stem to the P site whereas the peptidyl tRNA (the tRNA on which the peptide is formed) lies in a A/P hybrid state. To continue with the next cycle of elongation, translocation must happen so that the deacylated tRNA lies completely in the E site and the peptidyl tRNA in the P site. The translocation also creates an empty A site for entry of the next aa-tRNA. Translocation is an energy consuming process and requires the employment of eEF2 interaction which stabilises the hybrid state. eEF2-GTP hydrolysis induces a conformational change in the complex which promotes the translocation process (Fig. 1.12). This cycle continues for the entire length of the mRNA till a stop codon is reached (Ben-Shem et al., 2011; Dever and Green, 2012; Klinge et al., 2011).

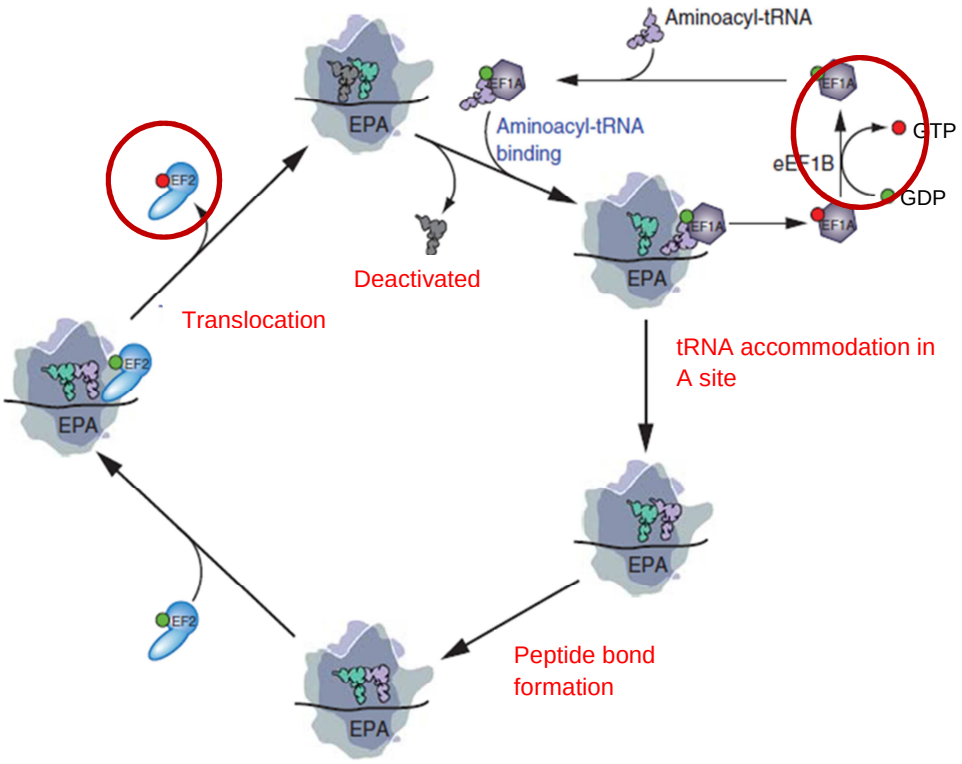



***Figure 1.12: Mechanism of translation elongation***

*The ternary elongation complex (eEF1A-GTP-aa.tRNA) binds to A site. The hydrolysis of GTP from eEF1A releases eEF1A from aa-tRNA. The amino acid on the aa-tRNA in the A site then forms a peptide with the amino acid in the P site. eEF2-GTP then promotes the translocation of tRNAs from A and P site into P and E site, respectively. The deacylated tRNA leaves the E site and the 80S subunit is ready to repeat the cycle of peptide elongation (adapted from Dever and Green, 2012).*

### 1.3.1 Regulation of elongation

Elongation could be regulated at two stages, viz. i) at the recycling of eEF1A-GDP to eEF1A-GTP, ii) by regulating the translocation of eEF2. The first mechanism is still unclear and hence yet to be studied (Kapp and Lorsch, 2004). Regulation of eEF2 can take place at two levels. eEF2 is a monomeric protein with a molecular mass of approximately 95 kDa. It has been shown that alterations at the diphthamide residue (a modified histidine found in eEF2) can alter the activity of eEF2. However, this mode of translation control still needs detailed studying. The other change in eEF2 that could lead to translation inhibition is the induction of phosphorylation of threonine residue at the position 56. Phosphorylation of eEF2 inhibits it from binding to the ribosome and hence hinders the translocation process. It was noted that this phosphorylation was calcium calmodulin dependant. Later studies revealed that eEF2 was phosphorylated by a dedicated kinase, eEF2K. eEF2K is a $Ca^{2+}$/calmodulin

dependant kinase. eEF2K undergoes auto-phosphorylation in a low calcium environment and phosphorylates eEF2. However, the mechanism is still unclear and needs further research (Dever and Green, 2012; Kapp and Lorsch, 2004; Kaul et al., 2011; Ryazanov, 1987).

## 1.4 Termination and recycling

The end of the elongation cycle is marked when the elongation complex encounters a stop codon so that the translation machinery enters the termination phase. The stop codons include UAA, UGA, and UAG. These three codons do not code for any amino acids and therefore function as stop codons. Termination is brought by the joint function of eukaryote release factors, eRF1 and eRF3. eRF1 recognises the stop codon and induces the release of the peptidyl tRNA. eRF1 resembles the tRNA and consists of three domains. The middle domain is equivalent to the acceptor stem of the tRNA and promotes the peptidyl release. eRF3 is a GTPase and interacts with the C terminus of the eRF1. The eRF1-eRF3-GTp ternary complex binds to the A site via the middle domain of the eRF1. This interaction leads to the hydrolysis of GTP and finally to the peptide release from the P site. The peptidyl release is also aided by ABCe1/Rli1 (Dever and Green, 2012; Kapp and Lorsch, 2004) (Fig. 1.13).

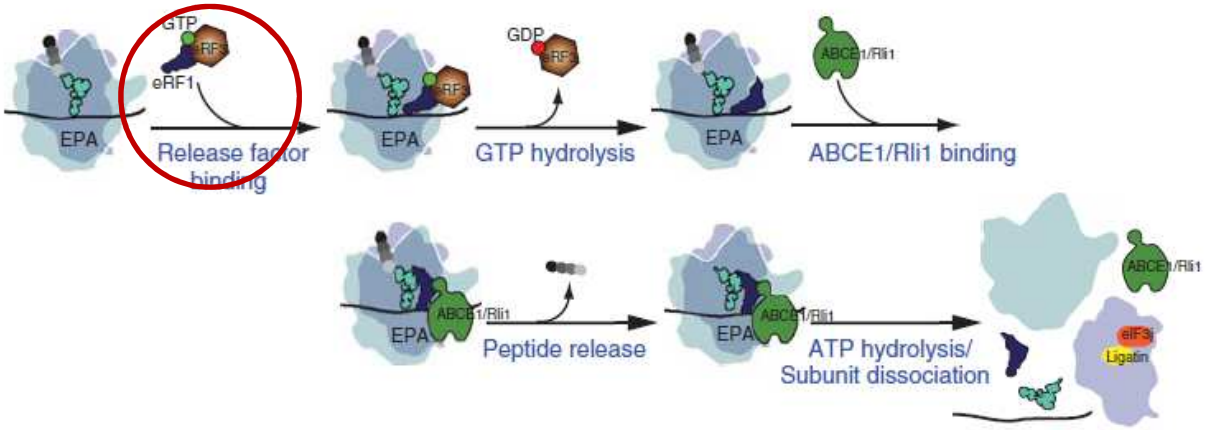



***Figure 1.13: Mechanism of translation termination***

*When the ribosome meets a stop codon, eRF1-eRF3-GTP binds to the A site which results in GTP hydrolysis to GDP and the release of eRF3. ABCE1/Rli 1 then interacts with the ribosome and releases the peptide (adapted from (Dever and Green, 2012)).*

The final stage in translation is the recycling of the ribosomal subunits back to the initiation phase. After the peptidyl release from the elongation complex, the ribosome is still bound to the deacylated tRNA in the P/E hybrid state, mRNA and eRF1. As

mentioned in Section 1.2.2, dissociation of 80S ribosome from the eRF1, tRNA and mRNA and the disjoining of the ribosome into 40S and 60 S subunits takes place with the help of eIF1, eIF1A and eIF3 (Korostelev et al., 2008; Weixlbaumer et al., 2008).

## 1.5 Eukaryotic translation still remains elusive

Though our understanding of the eukaryotic translation machinery has grown vastly in the last decade, it is quite clear that the eukaryotic translation machinery still requires to be studied. The scanning mechanism, the role of eEF1 and eEF2K are some examples where further studies are required (Kapp and Lorsch, 2004). As mentioned in Section 1, there is a dire need for the identification of new translation inhibitors and elucidation of their modes of action.

## 1.6 Small molecule translation inhibitors

Small molecule translation inhibitors have helped in elucidating the structure and function of the prokaryotic ribosome. They have also helped in dissecting the factors involved in translation. The lack of large number of small molecules that inhibit eukaryotic translation has limited our understanding of eukaryotic protein synthesis machinery (Chan et al., 2004). Small molecules can act as probes to elucidate the translation system. For example, rapamycin has helped us to understand the role of mTOR in translation. Likewise, pateamine A, a small molecule isolated from a marine sponge has helped in elucidating the role of eIF4A in the initiation process (Carlson, 2010; Low et al., 2005).

Natural products that inhibit translation also have been shown to be efficacious against cancer cells. Data from different studies show that various factors are dysregulated in cancer. As a result, translation inhibitors have been tested as anti-cancer agents and some of them have progressed to Phase II trials (*e.g.* homoharringtonine) (Stone et al., 2009).

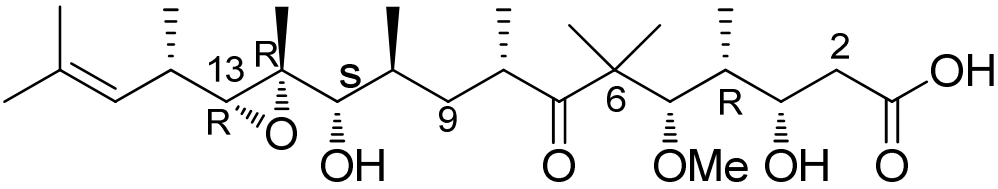
It has also been shown that viruses interact with the host translation system to synthesise their proteins. Therefore it has been suggested that targeting the host translation system could be used in an anti-viral strategy. For example, 4EGi-1 which interrupts eIF4F ribosome interactions has been shown to be antiviral against HSV-1 and VacV (McMahon et al., 2011).

Considering the above points it is important to study small molecules translation inhibitors and elucidate their mode of action. The next section will introduce four small

molecules which are mainly polyketides that target the eukaryotic translation system. These are: i) gephyronic acid. ii) tedanolide, iii) myriaporone 3/4, and iv) aetheramide B. The elucidation of the modes of action of these compounds is the main subject of this study.

### 1.6.1 Gephyronic acid

Gephyronic acid was isolated from the myxobacterial Archangium gephyra strain Ar3895. It showed cytotoxicity in the mammalian cell line L-929 in nanomolar ranges. It also inhibited the growth of different yeasts with a MIC of ~ 2µM and most filamentous fungi with a MIC of 50 µM. However, it did not show any activity against bacteria. Feeding experiments with radioactive precursors for thymidine representing DNA replication, uridine representing transcription and methionine representing protein synthesis showed that gephyronic acid induces cell death by inhibiting protein synthesis. However, the mechanism of translation inhibition by the polyketide was still unclear (Anderl et al., 2011; Nicolas et al., 2011; Sasse et al., 1995).

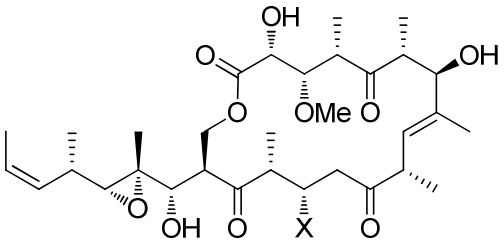



Gephyronic acid A

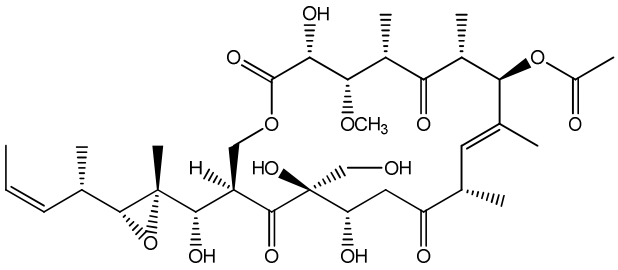

***Figure 1.14: Gephyronic acid A***

*The chemical structure of gephyronic acid A and the fruiting bodies of Archangium gephyra, the producing organism (photo curtesy Hans Reichenbach).*

### 1.6.2 Tedanolide




X=OH, tedanolide
X=H, 13-desoxytedanolide




Candidaspongiolide


***Figure 1.15: Structure of tedanolides***

*The structure of tedanolides which are e.g. produced by the sponge Tedinia ignis (photo courtesy: http://soundwaves.usgs.gov/2006/11/meetings2.html).*

Tedanolide was isolated from the marine sponge *Tedina ignis*, also referred to as fire sponge, which is found largely in the Caribbean region. Tedanolide is a 18-membered macrolactone containing propionate and acetate units. Initial studies reported tedanolide as potent cytotoxic macrolide with an $ED_{50}$ of 2.5 ng/mL in KB-3-1 cells. The study also reported that tedanolide arrested cells in the S phase of the cell cycle (Taylor, 2008). Later 13-deoxytedanolide was isolated from another sponge, *Mycale adhaerens* by Fusetani *et al. (1995),* which was even more potent showing an $IC_{50}$ of 94 pg/mL in murine leukaemia cells (Taylor, 2008). More recently, candidaspongiolide isolated from *Candidaspongia sp.* was reported (Meragelman et al., 2007). It differed from tedanolide in the region C11 to C15. Candidaspongiolide was cytotoxic with an $IC_{50}$ of 14 ng/mL in leukaemia cells. All these tedanolides were reported to inhibit translation in the eukaryotes but through different mechanisms. It was reported that interaction of 13-desoxytedanolide with the 60S subunit of the ribosome induces a ribotoxic stress which led to the translation inhibition (Lee et al., 2006). However, it

had been claimed that ribotoxic stress might not be completely responsible for the translation inhibition and further studies should be made to elucidate the mode of action (Trisciuoglio et al., 2008).

### 1.6.3 Myriaporone

Myriaporone 3/4 was isolated from the Mediterranean false coral *Myriapora truncata* (Fig. 1.16). The extracts from the false coral were shown to be toxic to cancer cells. Fractionation of the extracts led to the identification of myriaporone 3 and 4. The two isoforms were inseparable. Myriaporone 3/4 is it is a mixture of both isoforms (Hines et al., 2006).

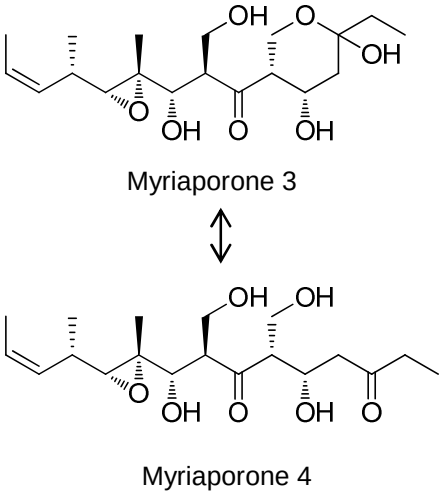


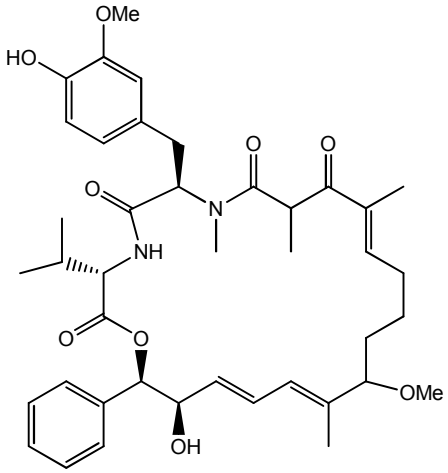


***Figure 1.16: The structure of myriaporone***

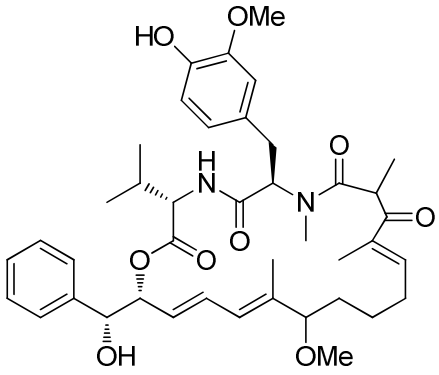
*The structure of myriaporone 3 and 4 which were isolated from the false coral Myriapora truncata (photo courtesy: http://coralmorphologic.com).*

Myriaporone 3/4 shows similarities in structure with tedanolide's "southern hemisphere". It has been reported that myriaporone 3/4 is a cytotoxic/cytostatic polyketide with an $IC_{50}$ of 15 nM in leukaemia cells. Like gephyronic acid and tedanolide, myriaporone 3/4 is not active in bacteria. The cytotoxicity was attributed to its activity as a translation inhibitor (Hines et al., 2006; Taylor, 2008). However, the mode of action of myriaporone was still unknown and yet to be studied.

### 1.6.4 Aetheramide B




Aetheramide A




Aetharamide B

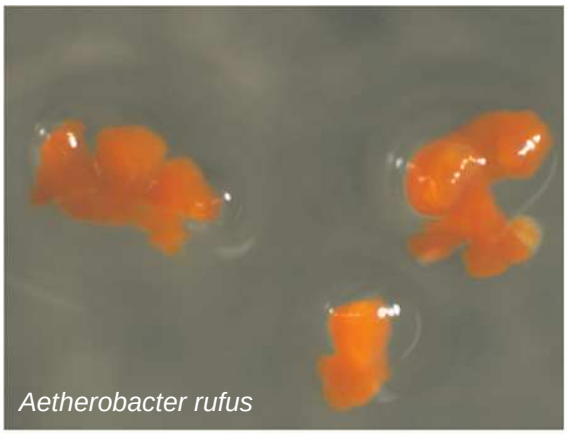



***Figure 1.17: The structure of aetheramides***

*The structure of aetheramide A and B and the fruiting bodies of Aetherobacter rufus, the producing organism (photo courtesy: Alberto Plaza).*

Aetheramide A and B were isolated from the new myxobacterial species *Aetherobacter rufus (Plaza et al., 2012)* (Fig. 1.17). It is not a pure polyketide because it has two amino acids built in, valine and a rare amino acid, 3-(4-hydroxy-3-methoxyphenyl)-2-(methylamino) propanoic acid (mNMeTyr) (Fig.1.17). Aetheramides showed an $IC_{50}$ of 110 nM in colon cancer cell lines. This thesis deals only with the identification of mode action of aetheramide B. During this study it was discovered that aetheramide B also is a translation inhibitor.

## 1.7 Aim of the project

The eukaryotic translation system is only partially understood and our current understanding is mostly a result of the extrapolation of the prokaryotic translation system (Poehlsgaard and Douthwaite, 2005; Schroeder et al., 2007; Tu et al., 2005). Translation is dysregulated in a number of diseases like cancer, Wolcott–Rallison Syndrome, during viral infections, etc. (Van Der Kelen et al., 2009). A better understanding of the translation machinery and the factors involved will help us design better drugs for therapeutics. Identification of new translation inhibitors specific to the eukaryotic system and elucidating the mode action of these compounds thus becomes very essential (Chan et al., 2004).

Gephyronic acid, myriaporone 3/4, des-epoxy tedanolide and aetheramide B are four compounds originally isolated from natural sources. The first three polyketides were reported as translation inhibitors earlier and aetheramide B was identified as a translation inhibitor in our routine screening processes.

This study aims at profiling the four translation inhibitors, identifying the molecular mechanism of translation inhibition induced by these compounds and identifying the possible direct targets in the translation system.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Equipment

| | |
|---|---|
| Bacterial incubator | Memmert |
| Cell culture incubator | $CO_2$-Auto-Zero (Heraeus) |
| Flow cytometer | BD LSR II (BD Biosciences) |
| Gel electrophoresis system | Mini PROTEAN system (Bio-Rad) |
| Semi-dry Transfer system | Biometra |
| X-ray processor | Optimax (ClassicXray) |
| Light microscopy | Axiovert 35 (Zeiss) |
| Fluorescence microscopy | Axioplan with Axiocam camera (Zeiss) |
| UV spectrophotometer | UV 1000 (Shimadzu) |
| Photometer | NanoDrop ND-1000 (NanoDrop technologies) |
| Plate reader | Infinite M200 pro (Tecan) |
| Centrifuges | Biofuge (Heraeus) |
| | 5810R (Eppendorf) |
| | 5418R (Eppendorf) |
| Laminar airflow | Maxisafe 2020 (Thermo Fisher Scientific) |
| Cell counter | Cedex XS (Innovatis) |
| Shaker | Titramax 1000 (Heidolph) |

### 2.1.2 Software

AxioVision 3.1, SigmaPlot 12.3, Microsoft Office 2010, ChemDraw, FACS Diva Version 6.1.1, ImageJ,

### 2.1.3 Consumables

All consumables were purchased from Becton Dickinson, Eppendorf, Greiner, Macherey-Nagel, Nunc, Thermo Fisher Scientific, TPP or Roth, unless otherwise stated.

### 2.1.4 Chemicals

All chemicals were obtained from Alexis Biochemicals, Bayer, Becton Dickinson, Fluka, Gibco, Invitrogen, JT Baker, Merck, Roche Diagnostics, Thermo Fisher Scientific, Roth or Sigma.

### 2.1.5 Antibodies/ Dyes

| | |
|---|---|
| Anti-eIF2α (mouse) | Cell Signalling |
| Anti-phospho-eIF2α (Ser52) (rabbit) | Invitrogen |
| Anti-4E-BP (rabbit) | Cell Signalling |
| Anti-phospho-4E-BP (Thr37/46) (rabbit) | Cell Signalling |
| Anti-mTOR (rabbit) | Cell Signalling |
| Anti-phospho-mTOR (Ser2448) (rabbit) | Cell Signalling |
| Anti-eEF2 (rabbit) | Cell Signalling |
| Anti-phospho-eEF2 (Thr56) (rabbit) | Cell Signalling |
| Anti-eEF2K (Ser366) (rabbit) | Cell Signalling |
| Anti-phospho-eEF2K (rabbit) | Cell Signalling |
| Anti-eIF4E (rabbit) | Epitomics |
| Anti-TIA (mouse) | Santa Cruz |

| | |
|---|---|
| Anti-mouse antibody (rabbit) | Dianova |
| Anti-rabbit antibody (goat) | Dianova |
| Alexa Fluor 488 anti-rabbit (goat) | Molecular Probes |
| Alexa Fluor 488 anti-mouse (rabbit) | Molecular Probes |
| Alexa Fluor 549 anti-rabbit (goat) | Molecular Probes |
| Alexa Fluor 549 anti-mouse (rabbit) | Molecular Probes |
| Alexa Fluor 488 streptavidin | Molecular Probes |

### 2.1.6 Culture media

For culturing bacteria EBS medium was used. For *Saccharomyces cerevisiae* YPD medium was used, for hyphal fungi M90 medium was used. *E.coli* was cultured in LB medium. All media components were purchased from Becton Dickinson, Fluka, Merck, Roth or Sigma.

| | |
|---|---|
| EBS (pH 7.0): | Peptone (5 g/L) |
| | Proteose peptone (5 g/L) |
| | Meat extract (1 g/L) |
| | Yeast extract (1 g/L) |
| | Yeast extract (1 g/L) |
| | HEPES (10 g/L) |
| M90 (pH 5.6): | Malt extract (30 g/L) |
| | Peptone (3 g/L) |
| LB (pH: 7.5, 1 L) | 10g Bacto-tryptone |
| | 5g yeast extract |
| | 10g NaCl |

For the agar media (agar diffusion tests), 15 g/L agar added. The medium was prepared in distilled $H_2O$ and autoclaved immediately. The autoclaved media were then stored at 4 °C. The mammalian cell culture media used are listed in Table 2.1

***Table 2.1: Media used for different cell lines***

| Cell line | Medium | Manufacturer | Supplements |
|---|---|---|---|
| **A-549, L-929** | MEM | Gibco | 10 % FBS (Lonza) |
| **KB-3-1, A-431** | DMEM | Lonza | 10 % FBS (Gibco) |
| **MCF-7** | DMEM | Gibco | 1X Non essential amino acids (Gibco)<br>1.5 % insulin (Gibco)<br>10% FBS (Lonza) |
| **A-498,PtK2** | MEM | Gibco | 1X Non-essential amino acids (Gibco)<br>1X GlutaMAX (Gibco)<br>10% FBS (Lonza) |
| **HUVEC** | EMB-2 | Lonza | EMB-2 bullet kit |
| **NHDF** | FGM-2 | Lonza | FGM-2 bullet kit |

### 2.1.7 Buffers

10 X SDS running buffer: 288 g glycine

60.4 g Tris Base

20 g SDS

1.8 L distilled water

The buffer was diluted to 1X with water to make the working running buffer.

10 X TBS buffer for western blotting (pH: 7.0): 160 g NaCl

4 g KCl

12.2 g Tris Base

2 L distilled water

The working TBS solution was prepared by diluting the 10 X buffer to 1X with water.

TBST buffer was made by adding 1 mL Tween-20 to 1000 mL of 1 X TBS buffer.

Blotting buffer: 25 mM Tris HCl

192 mM Glycine

### 2.1.8 Kits

| | |
|---|---|
| MycoAlert ® Mycoplasma Detection Kit | Lonza |
| Cell Titre Glo® ATP assay kit | Promega |
| Apoptosis detection kit I | BD Biosciences |
| Rabbit reticulocyte lysate (Nuclease treated) | Promega |
| Wheat germ extracts | Promega |
| E.coli cell extract | Promega |
| Pierce® Silver stain kit | Thermo Fisher Scientific |
| SuperSignal® West Pico Luminol solution | Thermo Fisher Scientific |

### 2.1.9 Microorganisms and Cell Cultures

***Table 2.2: Microorganisms***

| Microorganism | Abbreviations | Source |
|---|---|---|
| ***Escherichia coli tolC*** | TolC | Ciba-Geigy, Basel |
| ***Klebsiella pneumoniae*** | Kbp | HZI Collection |
| ***Pseudomonas aeruginosa*** | Psa | ATCC 9027 |
| ***Staphylococcus aureus*** | Sta | HZI Collection |
| ***Micrococcus luteus*** | Mcl | HZI Collection |
| ***Candida albicans*** | Caa | DSM 1386 |
| ***Hansenula anomala*** | Hna | DSM 70 263 |
| ***Saccharomyces cerevisiae BY4741*** | Scc | Euroscarf |
| ***Botrytis cinerea*** | Boc | DSM 877 |
| ***Pythium debaryanum*** | Pyd | DSM 62 946 |

***Table 2.3: Mammalian cell lines***

| Cell line | Source | Species | Origin | Morphology |
|---|---|---|---|---|
| **A-498** | DSMZ ACC 55 | Human | Kidney cancer | Epithelium-like |
| **A-549** | DSMZ ACC 107 | Human | Lung cancer | Epithelium-like |
| **NHDF** | Lonza | Human | Fibroblasts | Fibroblasts |
| **HUVEC** | Lonza | Human | Umbilical cord | Endothelium |
| **KB-3-1** | DSMZ ACC 158 | Human | Cervical cancer | Epithelium-like |
| **L-929** | DSMZ ACC 2 | Mouse | Connective tissue | Fibroblasts |
| **PtK2** | ATCC CCL-56 | Potoroo | Kidney | Epithelium-like |
| **U-937** | DSMZ ACC5 | Human | Lymphoma | Monocytes |

## 2.2 Methods

### 2.2.1 Working with microorganisms

#### 2.2.1.1. Handling

All microbiological studies were carried out under sterile conditions. The bacteria and yeasts were not maintained as a continuous culture. Whenever required, the cells were revived from frozen samples (-20°C). The hyphal fungi were stored at 4°C as spore suspension cultures. To start the bacteria and yeasts, a frozen culture was placed in fresh medium and shaken overnight at 30 ° C. The next day, the OD was determined at 600 nm using a photometer.

#### 2.2.1.2 Agar diffusion assay

For the agar diffusion tests complete media were used. For the bacteria EBS medium were used and for yeasts and hypal fungi YPD media and M90 media respectively. The medium (with agar) was heated in a microwave oven until the agar had melted and then cooled in a water bath at 50 ° C. The microorganisms were added to the medium at a final OD of 0.01 for bacteria and 0.1 for the yeasts. 15 mL of the inoculated media was then poured into Petri dishes and allowed to solidify. 20 µL of sample were applied to a filter paper disc (6 mm diameter) with a pipette under the laminar flow hood on a sterile glass plate and allowed to dry until the solvent had evaporated completely. Thereafter, the discs were placed on the agar plates with the microorganisms using forceps. The Petri dishes were then incubated at 30 ° C in a bacterial incubator for 1-2 days, depending on the speed of growth (usually 2 d for hyphal fungi and 1 d for bacteria). The diameter of inhibition zones was then measured.

### 2.2.2 Working with mammalian cell cultures

#### 2.2.2.1 Cultivation

The work with mammalian cell cultures was performed under sterile conditions. The required media were warmed at 37°C before using. The cells were kept in cell culture flasks at 37°C and 10% $CO_2$. The media volume depended on the size of the cell culture flasks viz. 10 mL for 25 $cm^2$, and 30 mL for 75 $cm^2$ bottles. Adherent cells like L-929 and KB-3-1 were harvested and passaged when they were subconfluent (~ 80-90% confluency). The cell layer was scraped with a cell scraper from the bottom of the vessel and the cells were suspended by repeatedly pipetting up and down with a sterile disposable plastic pipette. Other cell lines like MCF-7, PC-3, PtK2, A-431,

A-598 were trypsinised for passaging. To trypsinise the cells, the culture media was removed and the cell surface was rinsed once with EBSS followed by addition of 1 mL of trypsin. The flask was then incubated at 37°C for 5-10 minutes. The trypsinisation was stopped by adding fresh culture medium. Subsequently, an aliquot was transferred to a new cell culture flask with fresh medium.

All cell lines were maintained up to 1 year in culture. They were then discarded and a new culture from a cryo-preserve was restarted. All cell cultures were semi-annually tested for mycoplasma using MycoAlert® Detection Kits according to the manufacturer's protocol.

#### 2.2.2.2 Storage of cells

For long term storage, the cells were harvested as for sub-culturing. They were then harvested by centrifugation (3000 rpm, 3 min), pelleted and re-suspended in 1 mL of freezing medium. They were transferred to cryo-tubes which were put into a freezing container at -70 ° C for at least 24 hours. After that the cells were maintained in a liquid nitrogen storage container maintained at -196 ° C.

To reactivate the cryo preserved cells, the cells were quickly thawed at 37°C and seeded in 10 mL of fresh medium in a T25 flask. After one day, the cell culture medium was changed to remove the freezing medium completely.

### 2.2.3 MTT assay

Anti-proliferative activity was measured in 96-well plates. 60 µL of a serial dilution of the compound were added to 120 µL of suspended cells (50,000 cells/mL; two replicates). The end concentrations tested ranged from 37 µg to 0.2 ng/mL. After five days of incubation with the compound, the metabolic activity in each well was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). 20 µl MTT in PBS were added to a final concentration of 0.5 mg/mL and incubated for 2 hours. MTT is reduced then by dehydrogenases of the cells to form purple formazan crystals. The precipitate was washed with 100 µl PBS and dissolved in 100 µl isopropanol containing 0.4 % hydrochloric acid. The resulting colour was measured at 595 nm using a plate reader.

### 2.2.4 FACS analysis

FACS was used to measure apoptosis in cells. For this assay an Apoptosis Detection Kit from BD biosciences was used.

A-431 cells (~70% confluent) were treated with the inhibitors for 3, 6, 12 or 24 hours. Cells treated with methanol served as control. At the end of the incubation period, the cells were trypsinised, and resuspended in culture medium so that the final concentration of the cells was $1X10^6$. The cells were then centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The cell pellet was washed with 1 mL of ice cold PBS and centrifuged again. The supernatant was discarded and to the cell pellet was resuspended in 1 mL of the 1 X Binding buffer. 100 µL of this cell suspension was pipetted into a FACS tube and incubated in the dark for 15 minutes with 5 µL of FITC Annexin V and 5 µL of propidium iodide (PI). Then 400 µL of 1 X Binding buffer was added to make up the volume to 500 µL and the samples were analysed in a BD LSR II FACS machine.

### 2.2.5 *In vitro* translation assays

*In vitro* translation assays were performed with rabbit reticulocyte lysates, wheat germ extracts or *E.coli* cell lysates. All the kits were purchased from Promega.

#### 2.2.5.1 Rabbit reticulocyte lysate

Translation inhibition assay was performed by using the Flexi Rabbit Reticulocyte Lysate. Briefly, 500 ng of firefly luciferase mRNA was combined with 17.5 µL of rabbit reticulocyte lysate, 0.25 µL each of 1mM –Met and –Leu amino acid mixtures, 0.7 µL of 2.5 M KCl, 10 U RNasin, 3.3 µL of nuclease free water and 1 µL of the either gephyronic acid, myriaporone 3/4, des-epoxy tedanolide, aetheramide B or methanol to give final concentrations ranging from 100-0.001 µg/mL. Reactions were incubated at 30°C for 90 minutes. Luminescence was measured in a plate reader by mixing 2 µL of the mixture with 10 mL of the Firefly Luciferase Assay Substrate.

#### 2.2.5.2 Wheat germ extract

The manufacturer's protocol was modified a little to reduce the reaction volume. Briefly, 500 ng of firefly luciferase mRNA was combined with 12.5 µL of wheat germ extract, 2 µL of 1 mM –Met and –Leu amino acid mixtures, 3 µL of 1 M KCl, 40 U of RNasin, (1 µL from 40 U /µL), 3 µL of nuclease free water and 1 µL of either gephyronic acid, myriaporone 3/4, des-epoxy tedanolide, aetheramide B or methanol to give final concentrations ranging from 100-0.001 µg/mL. The reaction mix was

incubated at 25°C for 2 hours. Luminescence was measured in a plate reader by adding 2 µL of the reaction mix with 10 mL of the Firefly Luciferase Assay Substrate.

#### 2.2.5.3 *E.coli* S30 extract

The manufacturer's protocol was modified a little to reduce the reaction volume. Briefly, 1 µg of firefly luciferase control RNA provided in the kit was combined with 7.5 µL of S30 extract, 5 µL S30 Premix Without Amino Acids, 1.5 µL of 1 mM –Met and -Leu amino acid mixtures, 40 U of RNasin, (1 µL from 40 U /µL), 3 µL of nuclease free water and 1 µL of either gephyronic acid, myriaporone 3/4, des-epoxy tedanolide, aetheramide B or methanol to give final concentrations ranging from 100-0.001 µg/mL. The reaction mix was incubated at 25°C for 2 hours. Luminescence was measured in a plate reader by adding 2 µL of the reaction mix to 10 mL of the Firefly Luciferase Assay Substrate.

### 2.2.6 Cellular translation inhibition assay

The translation inhibition assay in cells was performed using a pRLSV40 vector (Promega). For the bicistronic reporter assays, the CrPV plasmid was purchased from Addgene and the polio IRES plasmid was a kind gift from Dr. Mario Koster, HZI. The assay consists of four parts: i) transformation, ii) plasmid purification, iii) transfection and iv) cellular translation inhibition assay

**Transformation:** Transformation was carried out to amplify the plasmid. This was done in competent *E.coli* xtBluc strain. The *strain* is maintained as a frozen culture at -80°C in cryo vials as 200 µL aliquots per vial.

The vials were thawed on ice and 10 ng of the plasmid to be amplified was added, mixed gently and incubated on ice for 30 minutes. The bacteria were heated at 42°C for 2 minutes and then placed on ice immediately for 5 minutes. The bacteria were transferred then to a test tube with 800 µL of LB Broth and incubated at 37°C for 60 minutes at 180 rpm. The culture was then transferred to Eppendorf tubes and centrifuged at 2000 g for ten minutes. The pellet was resuspended in 1 mL of LB Broth and 100 µL of this mix was plated on a LB-agar plate with ampicillin and incubated at 37°C overnight.

The next day a single colony was picked using a sterile toothpick. The toothpick was then dropped into a test tube with 5 mL of LB Broth with ampicillin and incubated at 37°C for 4 to 6 hours with shaking. At the end of the incubation period the bacterial culture was transferred to an one litre culture flask with 250 mL LB Broth and 250 µL

of 1 mg/mL ampicillin and incubated for 12 to 14 hours. Care was taken not to incubate them for more than 16 hours as this would lead to cell lysis.

**Plasmid purification:** The plasmid purification was carried out using NucleoBond Plasmid purification kit. The overnight bacterial culture was collected in a 250 mL tube and centrifuged at 4000 xg for 15 minutes. The supernatant was discarded and the pellet was gently resuspended in 12 mL of Buffer S1 supplemented with RNase. To this 12 mL of Buffer S2 was added, mixed by inverting gently 6 to 8 times and incubated at room temperature for 3 minutes. Then 12 mL of Buffer S3 was added and inverted 6-8 times till an off white flocculate is formed. The mix was then incubated on ice for 5 minutes. The NucleoBond column was equilibrated with 5 mL of N2 Buffer. The flow through was discarded. The flocculate formed was filtered using a filter paper and the filtrate was collected. The filtrate was then loaded onto the column and the flow through was discarded. The column was washed with 32 mL of N3 Buffer. The plasmid was eluted from the column using 15 mL of N3 Buffer. To this 11 mL of room temperature isopropanol was added to precipitate the plasmid. The mix was then transferred to a 50 mL glass centrifuge tube and centrifuged at 15000 x g for 45 minutes at 4°C. The supernatant was discarded and the pellet was dissolved in 1 mL of 70 % ethanol and centrifuged again at 15000 g for 10 minutes at 4°C. The ethanol was then discarded and the pellet was dried at 37°C till the ethanol evaporated completely. The transparent pellet was then dissolved in 150 µL of nuclease free water. The quantity and purity of the plasmid was measured using a Nanodrop ND 1000.

**Transfection:** Confluent KB-3-1 cells were trypsinised and cells were counted. The cells were resuspensed to get a final concentration of 1 X $10^5$ cells/mL. 100 µL of this cell suspension was plated in each well of a 96-well plate. The following day, the medium was exchanged with 100 µL of fresh medium and the cells were incubated at 37°C for one hour. In the meantime, 48 µg of the plasmid was mixed with 480 µL of DMEM and incubated for 5 minutes. To this mix 96 µL of 1 µg/mL polyethylenime (PEI) was added, mixed gently and incubated at room temperature for 15 minutes (or till the mixture becomes turbid). At the end of the incubation period of one hour, the 96-well plate was removed from the incubator and 85 µL of the media was aspirated from each well. Then 6.5 µL of DNA-PEI mix was added to each well and incubated for 30 minutes in the incubator. Then 100 µL of fresh media was added and incubated for another 48 hours.

**Translation inhibition assay:** The medium from the 96-well plate with the transfected cells was removed and fresh medium with the compounds in concentration range of 100-0.001 µg/mL was added to the cells. The plate was incubated for 3 hours. The cells were then lysed with 1 X Passive Lysis Buffer® (Promega). 5 µL of the cell lysate was mixed with 10 mL of the Luciferase Assay Buffer (Promega) and the luminescence was measured using a plate reader.

### 2.2.7 Wash out assays

Confluent A-431 cells were trypsinised and counted. 100 µL of 8 x $10^5$ cells/mL cell suspension was plated in each well of a 96-well plate. The following day, the medium exchanged with fresh medium with varying concentrations of the compounds or methanol and incubated for either 6 or 24 hours. After the incubation period, the culture medium containing the compound was removed and washed gently with EBSS. Fresh culture medium without compounds or methanol was added and cells were cultured for 4 days. Cells that were continuously cultured in media with compounds or methanol were also cultured for 4 days. These cells served as comparison parameter. At the end of the incubation period the metabolic activity in each well was measured with an MTT assay and compared to cells that were continuously treated with compounds and methanol.

### 2.2.8 Cell viability assay with CellTitreGlo®

The cytotoxic effects of gephyronic acid and myriaporone 3/4 on primary (NHDF) and cancer cells (KB-3-1) were measured using the CellTitre-Glo® assay (Promega). The ATP level is taken as a parameter of cell viability. 10,000 cells/well were seeded in a 96-well plate, grown over night and treated with serial dilutions of gephyronic acid or myriaporone 3/4. The final volume was maintained at 100 µL per well. Cells treated with methanol only served as control. After either 48 or 72 hours of incubation, the cells were mixed with 100 µL of CellTitre-Glo® and incubated at 37°C for 30 minutes. The plates were then left at room temperature in dark or wrapped in aluminium foil to normalise. The plate was shaken on a shaker for one minute and centrifuged shortly. The luminescence which correlates to the ATP levels in the cell were measured using a plate reader.

### 2.2.9 Angiogenesis

Inhibition of tube formation *in vitro* was tested using human umbilical endothelial cells (HUVECs) from LONZA. Frozen Matrigel (BD Biosciences) was thawed at -4°C

overnight, and after mixing it with an equal volume of cold EBM 2 medium (LONZA), 35 µL aliquots were distributed in wells of a 96-well plate placed on ice. The plates were kept at 37°C then for 1 hour to let the Matrigel solidify. 25 µL of a suspension of HUVECs (8 x $10^5$ cells/mL) that were harvested from a grown flask by trypsinization were given to each well, to which serial dilutions of the compounds were added. Plates were incubated at 37°C and 10 % $CO_2$ overnight, and the tube formation was judged under the microscope with a 5x lens and documented using an attached CCD camera.

### 2.2.10 Immunofluorescence

**Cell seeding:** For fluorescence staining, cells were cultured on coverslips. First, a sterile coverslip with a diameter of 13 mm was placed into a well of a four well plate. PtK2 cells harvested by scrapping of a semi-confluent culture were diluted with medium 1:3. 750 µL of the diluted cell suspension was added to each well. and incubated overnight to ensure complete adherence. The compounds were added the following day. Care was taken that the concentration of the solvent MeOH and DMSO did not exceed 1%. The cells were incubated with the samples, for 4 to 6 hours depending on the experimental requirement at 37°C.

**Fixation:** After the incubation, the cells were fixed with 750 µL of 3.7 % formalin for 10 minutes The cells were washed once with sterile PBS and then treated for 5 min with 0.1% Triton X-100. In the treatment with formalin, the proteins are cross-linked and fixed in a gentle manner. Triton X-100 permeabilized the cells, allowing the antibodies / dyes to reach into the interior of the cells. After Triton X-100 treatment, the cells were also washed once with sterile PBS.

**Staining:** Staining was done with appropriate antibodies. The primary antibody dilution varied with the antibody in use. Usually it was in the range of 1:50 to 1:500. The dilutions were prepared in PBS containing 10% FBS. DAPI was diluted to 1 ug / mL in PBS. The cells were incubated for 60 minutes with 250 µL of the primary antibody at 37°C and then washed with PBS twice. Then the cells were incubated with 250 µL of secondary antibody tagged by a fluorochrome (Alexa Fluor 488 or Alexa Fluor 594). The secondary antibody was diluted to 1:500 with PBS containing 10 % FBS. The cells were washed twice with PBS. The coverslips were then removed from the wells, towel dried and embedded (with the cells down) onto a microscope slide with a drop of embedding medium (ProLong Antifade).

**Creating the images:** Images were taken with the CCD camera Axiocam mounted on the fluorescence microscope Axioplan (both from Zeiss). Neofluar objectives with magnifications of 63X and 100X were used for viewing. All the images were taken at 63X magnification.

### 2.2.11 SDS-PAGE

SDS PAGE was performed using a commercial available gel from Bio Rad (4-20% Tris HCl gradient gel). Cells were lysed using MPER cell lysis buffer from Thermo Fisher Scientific supplemented with HALT protease/phosphatase inhibitor from Roche (1X). Protein was quantified using the Bradford method.

40 µg of protein from the lysate was mixed with 5 µL of 4X SDS loading dye (Roth) and heated at 96°C for ten minutes. The sample was then loaded to the individual wells of the SDS gel placed in a vertical electrophoresis tank with SDS running buffer. A marker was also loaded to one well. Electrophoresis was carried out at 120 V for 1.5 hours or till the loading front reached the end of the gel.

### 2.2.12 Western blot

**Transfer**: The proteins from the SDS PAGE gel were transferred to nitrocellulose membranes by a semi dry western blot method. The nitrocellulose membrane and Whatman filter paper was cut into 6 x 9 cm pieces. The membrane and 6 Whatman filter papers were then soaked in 1X blotting buffer with 20% methanol for at least ten minutes. Then membrane was placed onto 3 layers of Whatman filter paper. The gel was layered over the membrane and 3 more layers of Whatman paper were placed. The transfer was allowed to proceed at 120 V and 80 mA for 1 hour.

**Blocking and antibody incubation**: The membrane was then removed from the transfer cassette and incubated in 5 % non-fat milk prepared in TBST buffer for 2 hours at room temperature to block unspecific binding sites. The membrane was then washed shortly once with TBST. 10 µL of the stock antibody was added to 10 mL of TBST solution containing 1% BSA. This antibody mix was added to the blot and the blot was incubated at room temperature for 2 hours or at 4°C overnight. The primary antibody solution was removed, and washed 3 times with TBST. The blot was then incubated with 10 mL TBST containing 1% BSA and 0.5 µL of secondary antibody conjugated to HRP. The membrane was washed with TBST again and prepared for detection.

**Detection**: Supersignal West Pico® chemiluminescence detection kit (Thermo Fisher Scientific) was used for detection. The membrane was incubated with a mix of Enhancer and Stable Peroxide Buffer in equal parts for 1 to 5 minutes. The membrane was then transferred to an X-ray cassette. In the dark room an X-ray film was placed in the cassette and exposed for 0.5 to 5 minutes. The film was developed using an Optimax developing machine.

### 2.2.13 Silver staining

Silver staining was carried out with a Pierce Silver Stain Kit from Thermo Fisher Scientific. The SDS gel was removed from the electrophoresis cassette and washed twice for 5 minutes in filtered Milipore water to remove the salts of the running buffer. Then the gel was incubated for 15 minutes in fixing solution made of 30% ethanol,10% acetic acid and 70 % water. The fixing process was repeated once again for 15 minutes. Then the gel was washed twice for 5 minutes each with 10% ethanol and twice with Milipore water for 5 minutes each. In the meanwhile the Sensitizer Working Solution was prepared (50 µL Sensitizer mixed with 25 mL water).The gel was incubated with the Sensitizer Working Solution for 1 minute, followed by 2 washes with water for 1 minute each. The Stain Working Solution (0.5mL Enhancer mixed with 25 mL Stain) was prepared and the gel was incubated for 30 minutes. The Developer Working Solution (0.5 mL Enhancer with 25 mL Developer) was prepared. The gel was washed with ultrapure water twice for 20 seconds at each wash. The gel was then incubated with the Developer Working Solution 2 to 3 minutes until the bands appeared. Immediately after the bands appeared 5% acetic acid was added and incubated for 10 minutes.

### 2.2.14 Pull down assay

**Preparation of cell lysates:** KB-3-1 cells were cultured in three 75-$cm^2$ flasks till they reached a 60-70% confluency. The culture medium was discarded and washed once with EBSS. The cells were then trypsinised, pooled, centrifuged and the cell pellet was collected. The cells were lysed with 1 mL of MPER lysis buffer supplemented with 1X Halt protease-phosphotase inhibitor on ice for ten minutes. The lysate was centrifuged at 10,000g for ten minutes at 4°C. The supernatant was collected and the protein measurement measured using Bradford's assay. The total protein concentration was maintained between 6-8 mg/mL.

**Affinity capture of the target protein:** The cell lysate was split into four Eppendorf tubes each of 200 µL in volume. 2 µL of methanol was added to tube 1, 2 µL of biotinylated gephyronic acid to tube 2 (1 mg/mL concentration), 1 µL of biotinylated gephyronic acid to tube 3 and 2 µL of gephyronic acid to tube 4.All the tubes were incubated on ice with mild shaking for 1 hour. Then 1 µL of gephyronic acid was added only to tube 3 and all tubes were incubated at 4°C for another 1 hour.

At the end of one hour, the contents of the individual tubes were loaded to different 1.5-mL Eppendorf tubes with 200 µL Dynabeads® M-280 Streptavidin beads from Invitrogen. The beads were washed 3 times with PBS before the lysates were added. The lysate-Dynabeads mix was incubated for 1 hour at room temperature with intermediate shaking. After one hour the Eppendorf tubes were centrifuged at 5000 rpm for 2 minutes to sediment the streptavidin coated magnetic beads. The supernatant was discarded and the beads were washed 3-4 times with PBS to remove unbound and unspecifically bound proteins. The beads were then mixed with 40 µL of 4X SDS loading buffer from Roth and heated at 96°C for ten minutes. The tubes were cooled on ice and centrifuged at 5000 rpm for 5 minutes. The collected supernatant contained the biotinylated proteins from the cell lysate and the target of gephyronic acid A.

**Detection:** 20 µL of the heated loading dye with proteins were then loaded in the wells of 4-20% gradient SDS gel and electrophoresis was performed as mentioned above. The gel was then subjected to either silver staining or western blotting.

### 2.2.15 DARTS

**Preparation of cell lysate**: KB-3-1 cells were cultured to 70-80% confluency in a 75-$cm^2$ cell culture flask. The cells were trypsinised and centrifuged. The cell pellet was washed once with PBS and centrifuged again. The pellet was resuspended in 800 µL of MPER lysis buffer (Pierce) in a 1.5-mL Eppendorf tube and subjected to cell lysis on ice for ten minutes. After ten minutes, the tubes were centrifuged, the supernatant was collected and the protein content measured using Bradford's assay.

**Digestion with pronase:** 40 µg of the protein lysate was incubated with the compounds or methanol for 2 hours at room temperature. The mixture was digested with 20 ng of Pronase (Roche) at 37°C for 30 min. The digestion was stopped by heating with 5 µL 4X loading dye (Roth).

**Detection:** The sample was separated by SDS-PAGE (4-20% Tris-HCl gels) and was either subjected to Coomassie staining and viewed or transferred onto a nitrocellulose membrane. The membrane was then subjected to western blotting with the required antibodies and viewed by the chemiluminescene as mention in section 2.2.12.

### 2.2.16 Overexpression assay

Translation inhibition assay was performed by using the Flexi Rabbit Reticulocyte Lysate system (Promega) supplemented with either with an eEF2K expressed lysate or empty vector lysate (Novobios). Briefly, 500 ng of luciferase mRNA was combined with 17.5 µL of rabbit reticulocyte lysate, 0.25 µL of 1 mM –Met and –Leu amino acid mixtures, 0.7 µL of 2.5 M KCl, 10 U RNasin, 3.3 µL of nuclease free water, 25 µL of either eEF2K or empty vector lysate (containing 100 µg/mL of total protein) and 1 µL of myriaporone to give a final concentration ranging from 100-0.001 µg/mL. The reaction mixtures were incubated at 30°C for 90 minutes. Luminescence was measured by mixing 2 µL of the mixture with 10 mL of the Luciferase Assay Substrate (Promega).

# 3 Results

## 3.1 Establishment of polyketides as translation inhibitors

### 3.1.1 Cytotoxicity profiling with mammalian cells

To test the cytotoxicity of the four polyketides that were investigated during this study in higher eukaryotes different mammalian cell lines were used, including the human cancer cell lines KB-3-1, PC-3, A-459, and A-431, and transformed cells from mouse and potoroo, L-929 and PtK2, respectively. The compounds were incubated with the cells in serial dilutions in micro-titre plates for a period of 5 days. The influence on the metabolic activity of the cells was measured by an MTT assay. As can be seen from Table 3.1 all the four compounds inhibited the viability of the cells in nanomolar ranges.

***Table 3.1: Cytotoxicity of the polyketides in mammalian cells***

*The values represent the $IC_{50}$ of the each compound measured by an MTT assay.*

| Cell line | Gephyronic acid (nM) | Myriaporone 3/4 (nM) | Des-epoxy tedanolide (nM) | Aetheramide B (nM) |
|---|---|---|---|---|
| L-929 | 100 | 8 | 1.6 | 40 |
| KB-3-1 | 14 | 4 | 0.6 | 30 |
| A-431 | 14 | 11 | 1.2 | - |
| A-549 | 8 | 10 | - | 35 |
| PC-3 | 6 | 8 | 5 | 15 |
| PtK2 | 32 | 10.5 | 1.8 | 30 |

### 3.1.2 Antimicrobial activity profiling with prokaryotes and fungi

All four polyketides were examined for their biological activity towards a broad spectrum of microorganisms. By default, eight microorganisms (bacteria and fungi) were tested. Ciprofloxacin, a known antibiotic, and nystatin, a known antifungal agent were used as positive controls and also as a comparison standard. The profiling was carried out using zone of inhibition assays as explained in the methods section. The results are depicted in Table 3.2.

***Table 3.2: Results of agar diffusion assays with microorganisms***

*Diameter of zone of inhibition is given in mm.*

| **Test organism** | Gephy-ronic acid | Myria-porone 3/4 | Des-epoxy tedanolide | Aetheramide B | Cipro-floxacin | Nystatin |
|---|---|---|---|---|---|---|
| Gram-negative Bacteria | | | | | | |
| *E. coli tolC* | < 7 | < 7 | < 7 | < 7 | 16 | < 7 |
| *Klebsiella pneumoniae* | < 7 | < 7 | < 7 | < 7 | 13 | < 7 |
| *Pseudomonas aeruginosa* | < 7 | < 7 | < 7 | < 7 | 27 | < 7 |
| Gram-positive Bacteria | | | | | | |
| *Staphylococcus aureus* | < 7 | < 7 | < 7 | < 7 | 31 | < 7 |
| *Micrococcus luteus* | < 7 | < 7 | < 7 | < 7 | 40 | < 7 |
| *Yeasts* | | | | | | |
| *Candida albicans* | 27 | 20 | 16 | 10 | < 7 | 19 |
| *Hansenula anomala* | 30 | | 16 | < 7 | < 7 | 25 |
| *Saccharomyces cerevisiae* | 21 | 15 | < 7 | < 7 | < 7 | 20 |

### 3.1.3 Translation inhibition *in vitro*

Three of the four polyketides (gephyronic acid, myriaporone 3/4, des-epoxy tedanolide) had been shown to inhibit translation previously. To confirm these findings and to determine the $IC_{50}$ values in translation inhibition, *in vitro* translation assays were performed. A rabbit reticulocyte lysate was supplemented with firefly luciferase mRNA and amino acids and incubated with different concentrations of the test compounds. The resulting luminescence which is directly proportional to the translation of luciferase mRNA was measured after 90 minutes of incubation at 30°C. The readout showed an inhibition of translation by all compounds. The $IC_{50}$ values were 0.07 µM for gephyronic acid (Fig. 3.1A), 0.4 µM for myriaporone 3/4 (Fig. 3.1B), 0.01 µM for des-epoxy tedanolide (Fig. 3.1C) and 1.1 µM for aetheramide B (Fig. 3.1D). Cycloheximide, a known eukaryotic translation inhibitor was used as a positive control. It showed an $IC_{50}$ of 1.5 µM.

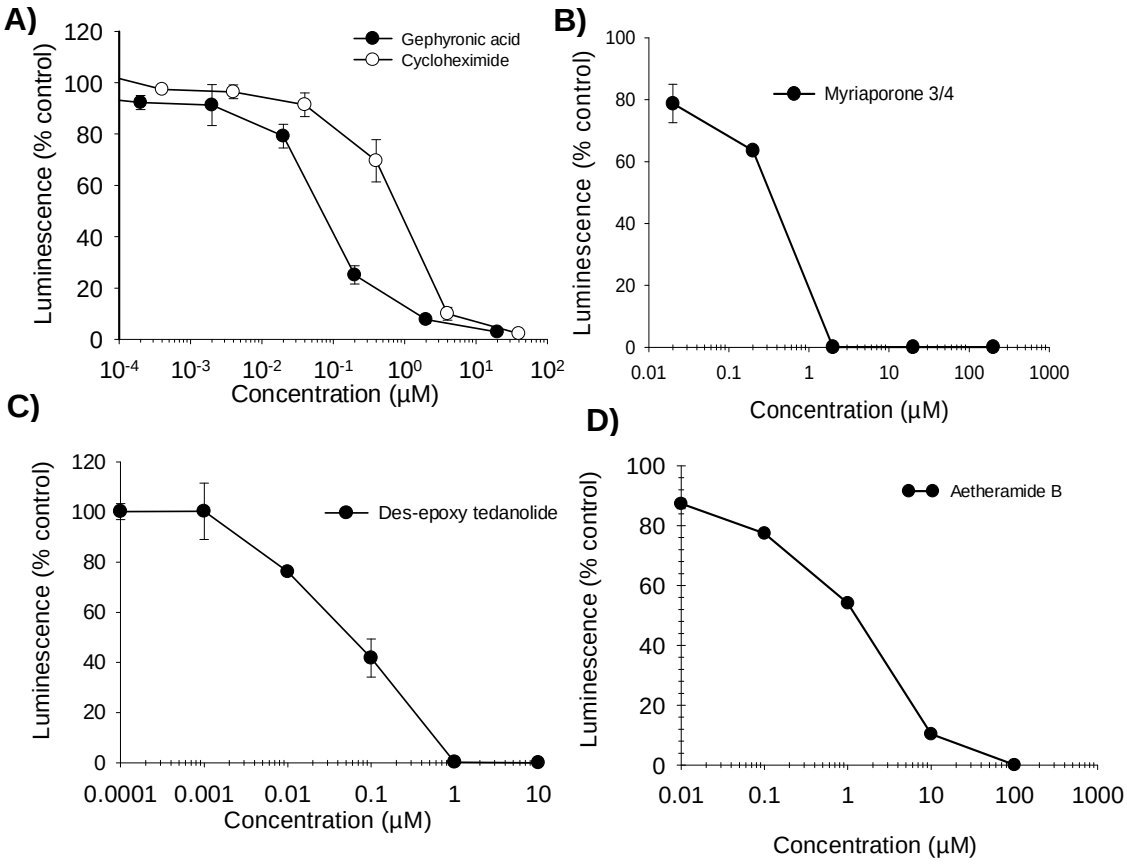



***Figure 3.1:Translation inhibition in rabbit reticulocyte lysates***

*Translation inhibition in rabbit reticulocyte lysates by gephyronic acid (A), myriaporone 3/4 (B), des-epoxy tedanolide (C) and aetheramide B (D). Cycloheximide was used as a positive control (A).*

### 3.1.4 Translation inhibition in cells

To prove the inhibition of de novo protein synthesis in cells, KB-3-1 cells were transfected with a pRLSV40 vector coding for Renilla luciferase. 48 hours after transfection, cells were treated with varying concentrations of either gephyronic acid, myriaporone 3/4, des-epoxy tedanolide, aetheramide B or cycloheximide for 4 hours. Cycloheximide inhibited the cellular translation with an $IC_{50}$ of 50 nM. Gephyronic acid showed an $IC_{50}$ of 30 nM, myriaporone 3/4 of 100 nM, des-epoxy tedanolide of 0.2 nM and aetheramide had an $IC_{50}$ of 700 nM (Fig. 3.2). These results were consistent with the *in vitro* data.

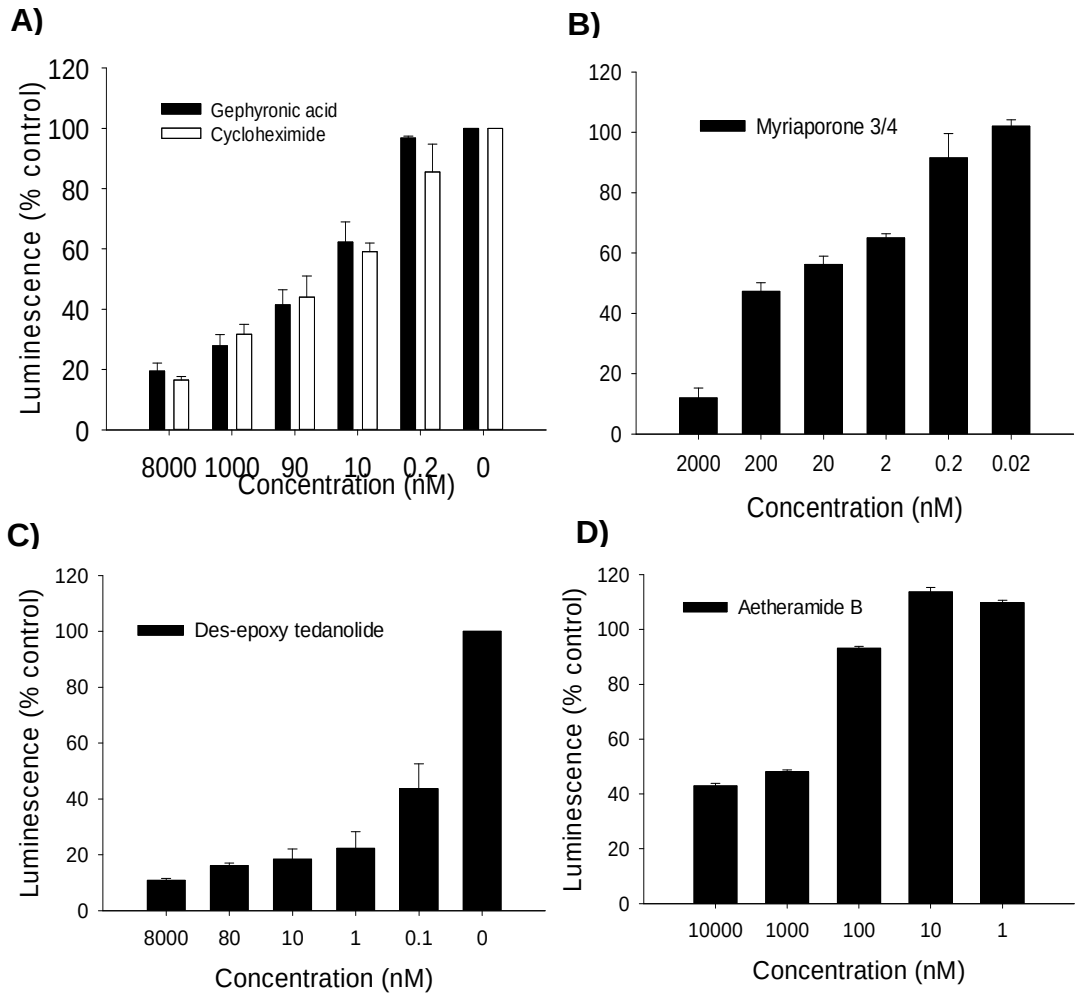



***Figure 3.2: Translation inhibition in KB-3-1 cells***

*Translation inhibition in KB-3-1 cells transiently transfected with pRLSV40 vector by gephyronic acid (A, black bars), myriaporone 3/4 (B), des-epoxy tedanolide (C) and aetheramide B (D). Cycloheximide was used as a positive control (A, white bars).*

## 3.2 Identification of the mode action of gephyronic acid

The results reported in the previous section showed that all the four compounds inhibited protein biosynthesis. This could be proven by assays in vitro and in cell cultures. In the following the mode of action of each compound is described in detail.

### 3.2.1 Induction of apoptosis

To test for the induction of apoptosis by gephyronic acid, A-431 skin cancer cells were treated with 50 nM of the compound for 3, 6, 12 and 24 hours and labelled by PI and FITC-Annexin V. An ongoing apoptosis in the cells was measured by flow cytometry (FACS). Methanol treated cells were used as control. Fig. 3.3 shows the distribution of the fluorescent cells in 4 quadrants. Cells in quadrant 4 show early apoptosis, and cells in quadrant 2 late apoptosis. It can be seen that within 3 hours of treatment with gephyronic acid, a significant higher percentage of cells (in total 11 %) underwent apoptosis as compared to the control (4.4 %). After 24 hours the percentage has increased to 20 %.

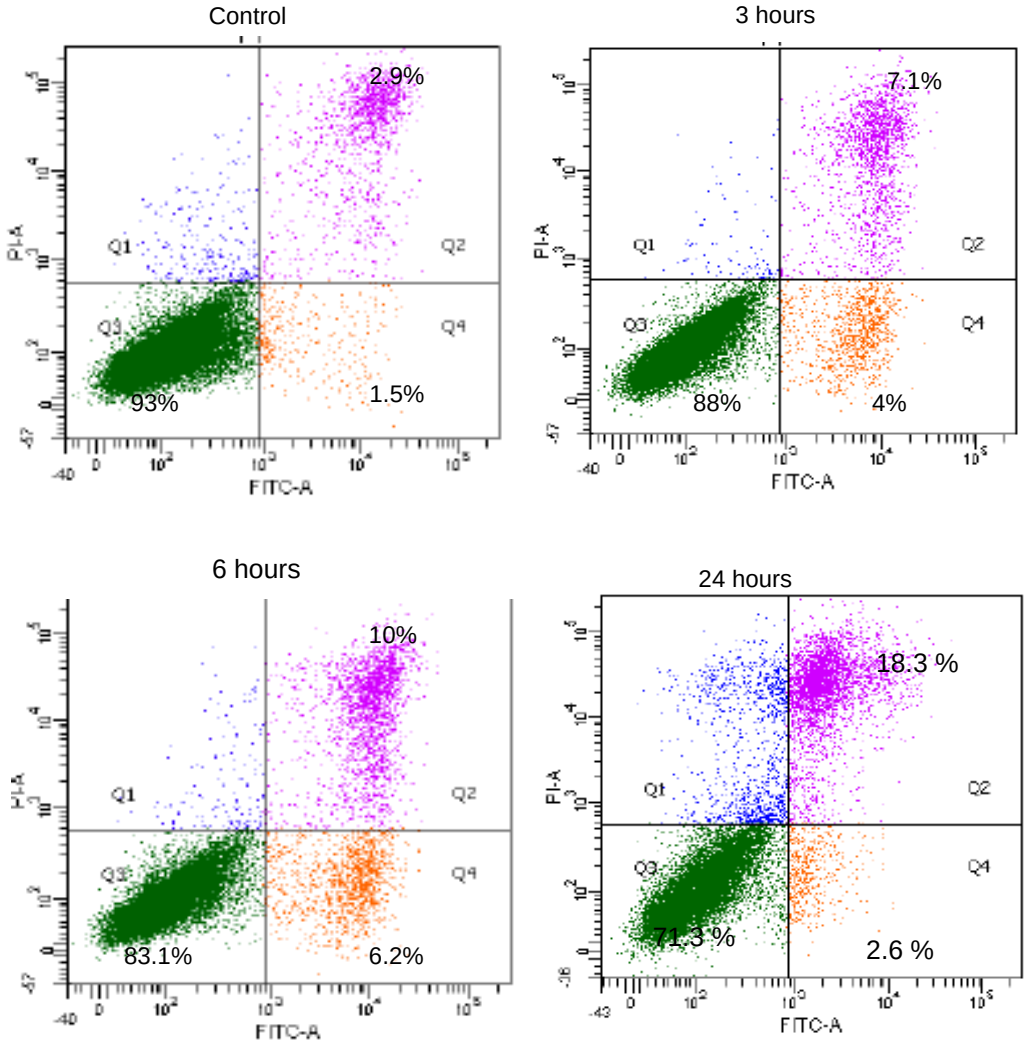



***Figure 3.3: Induction of apoptosis***

*FACS analysis of A-431 cells at different time points showed an increase in apoptotic cells when incubated with gephyronic acid. After 24 hours 20.9% of the cells treated with the translation inhibitor (50 nM) had undergone apoptosis compared to 4.5 % of the cells treated with methanol only.*

### 3.2.2 Reversibility of the cytotoxic effect induced by gephyronic acid

To test the reversibility of the cytotoxic effect of gephyronic acid, A-431 cells were treated with the inhibitor for 6 or 24 hours. At the end of the incubation period, the culture media of a part of the cells were removed and replaced by fresh media. The cells were cultured for further 4 days then. An MTT assay was performed to measure cell viability. As can be seen from Fig. 3.4, the $IC_{50}$ of the cells treated for 6 hours only was higher than of the control cells which were continuously treated with gephyronic

acid. After 24 hours the cytotoxic effects were no longer reversible. The $IC_{50}$ value of washed and non-washed cells differed only slightly.

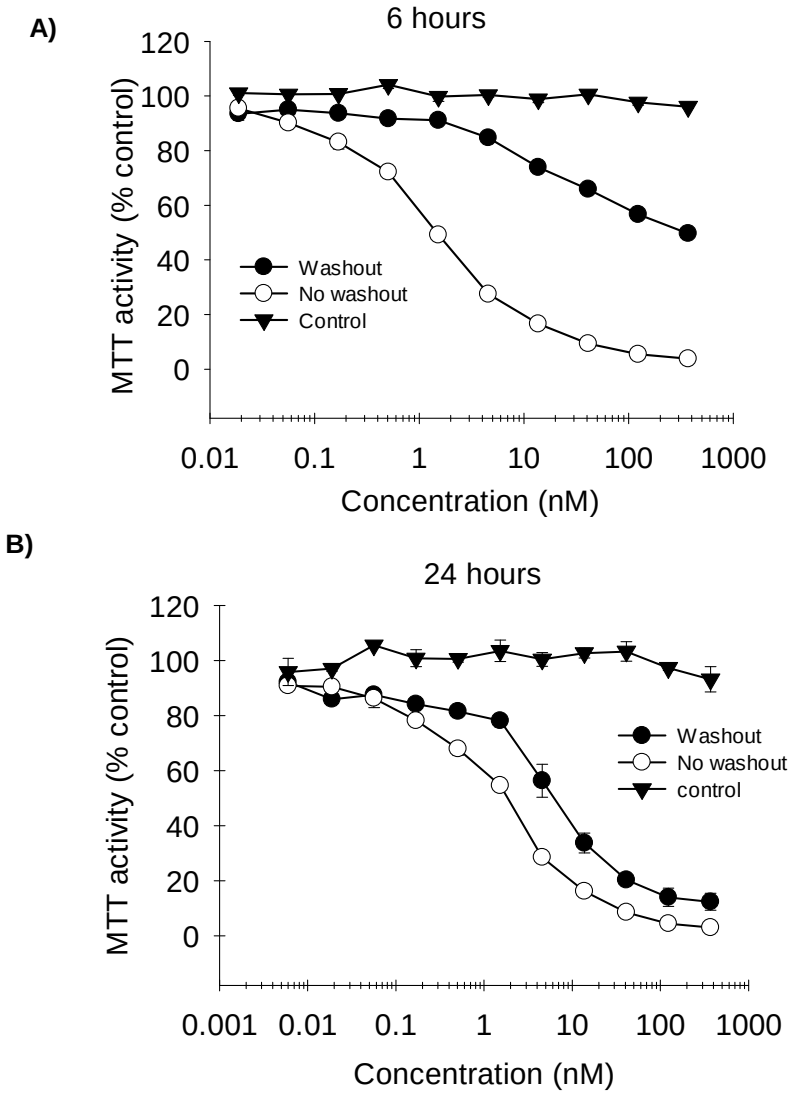



***Figure 3.4: Reversibility of the cytotoxic effect***

*Human epidermoid cancer cells A-431 were incubated with serial dilutions of the compound for 6 (A) or 24 (B) hours, washed with EBSS, and grown for further 4 days. Reference cells were continuously incubated with gephyronic acid. Cell viability was measured with an MTT test.*

### 3.2.3 Toxicity of gephyronic acid in primary cells

Translation inhibitors might have differences in cytotoxicity in transformed cells when compared to the primary, healthy cells. To test if this was true for gephyronic acid, cancerous KB-3-1 cells and primary dermal fibroblasts (NHDF) were treated with varying concentrations of gephyronic acid and analysed for their ATP content as a parameter of viability. After 72 hours of incubation, the $IC_{50}$ was 14 nM with KB-3-1 cells compared to 8 µM with NHDF (Fig. 3.5B). After 48 hours the $IC_{50}$ was 0.004 µM

with KB-3-1 cells and 2 µM for NHDF cells. (Fig. 3.5A). The results showed that the cancer cells were at least 80 fold more sensitive to gephyronic acid than normal cells.

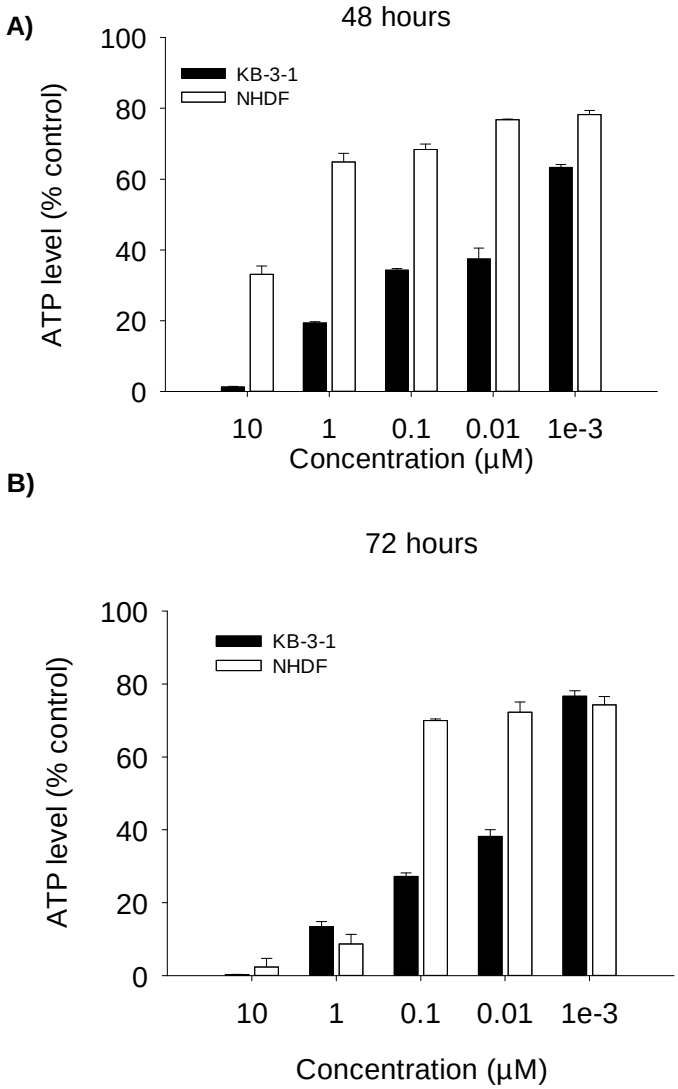



***Figure 3.5: Comparison of the toxicity of gephyronic acid to healthy and cancer cells***
*ATP level were measured as a parameter of viability with KB-3-1 and NHDF cells. Gephyronic acid induced less cytotoxic effects in primary NHDF cells.*

### 3.2.4 In vitro tube formation as a model for angiogenesis

Translation inhibitors like rapamycin, have been shown to inhibit angiogenesis, i.e. translation inhibitors are anti-cancer agents (Del Bufalo et al., 2006). To test the potential of gephyronic acid in that respect an *in vitro* tube formation assay was used. HUVECs were seeded on Matrigel®, which mimics the angiogenesis *in vivo*. While the control cells formed vessel like structures, cells treated with gephyronic acid did

not form tubes (Fig. 3.6). This effect was dose dependant. The minimal inhibition concentration (MIC) was observed to be 0.3 µM.

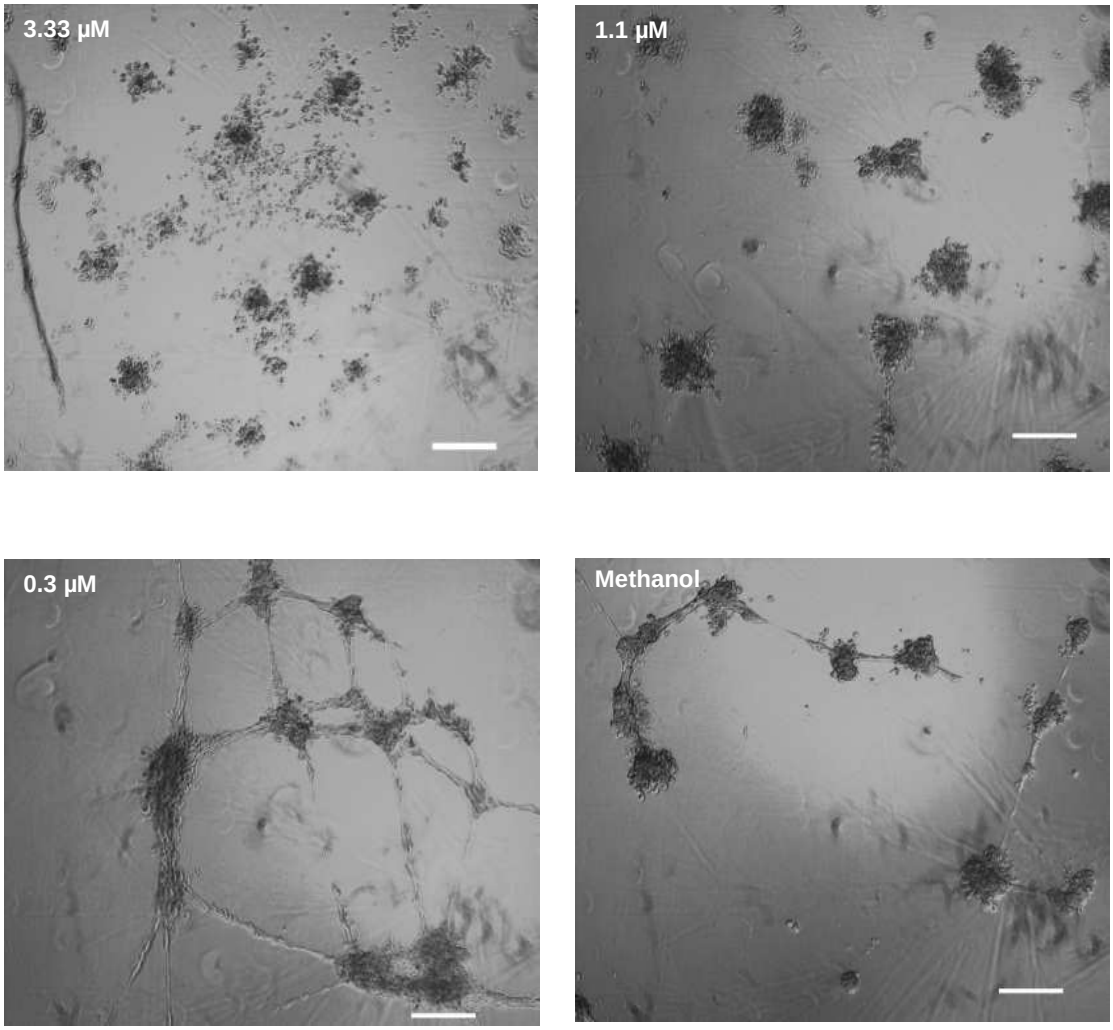



***Figure 3.6: Gephyronic acid inhibits tube formation in vitro***

*HUVECs plated on Matrigel were treated with various concentrations of gephyronic acid. Gephyronic acid inhibited angiogenesis in a dose dependant manner with an MIC of 0.3 µM.*

### 3.2.5 Comparison of inhibition in eukaryotic and prokaryotic translation systems

Gephyronic acid inhibits translation in the rabbit reticulocyte lysate system. The next assessment was to analyse if gephyronic acid was a pan eukaryotic translation inhibitor. Therefore wheat germ lysate supplemented with luciferase mRNA and amino acids was employed. The mixture treated with various concentrations of gephyronic acid was incubated at 37ºC for 60 minutes and the luminescence was measured. As can be seen from Fig. 3.7A, gephyronic acid inhibited translation in wheat germ lysate

in a dose dependant manner showing that gephyronic acid is a general eukaryotic translation inhibitor. However, when gephyronic acid was tested with *E. coli* lysate supplemented with luciferase mRNA, there was no inhibition of luminescence as seen from Fig. 3.7B.

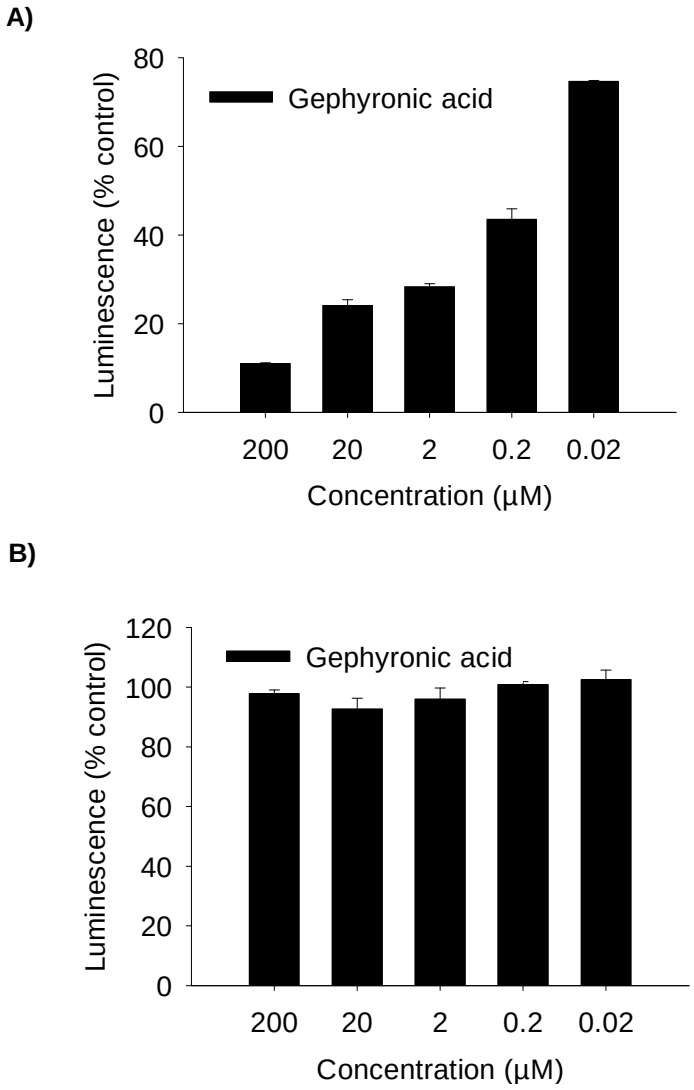



***Figure 3.7: Gephyronic acid does not inhibit prokaryotic translation***

*Wheat germ lysate (A) and E.coli cell lysate (B) were incubated with luciferase mRNA, amino acids and gephyronic acid at various concentrations. The luminescence plotted with respect to control shows a dose dependent reduction in translation only in wheat germ lysate.*

### 3.2.6 Determining the phase of translation inhibition

To determine the phase of translation inhibition induced by gephyronic acid, a bicistronic system which consisted of firefly luciferase translated by a cap-dependant system and Renilla luciferase translated by CrPV or polio IRES system was used (Fig. 3.8).

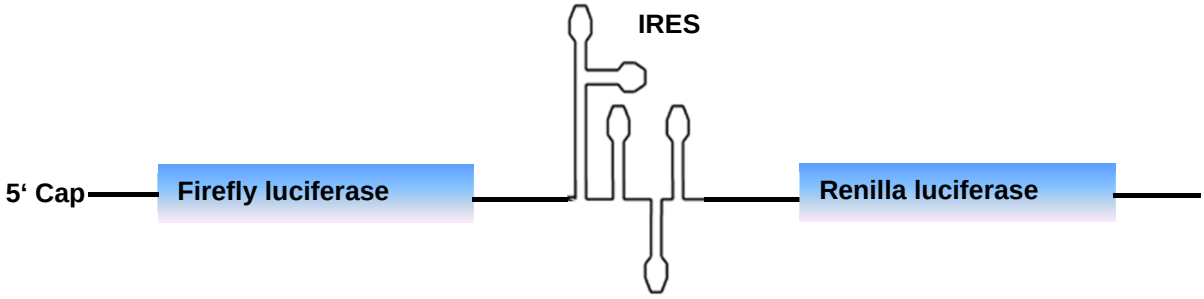



***Figure 3.8: Structure of the bicistronic vector***

*The bicistronic vector consists of two open reading frames (ORF). The first ORF coding for the firefly luciferase is translated via the 5’ cap dependant system and the Renilla luciferase is translated by the IRES system.*

KB 3-1 cells transfected with the CrPV bicistronic vector were treated with gephyronic acid, cycloheximide and pateamine A at concentrations ranging from 1.0 - 0.01 µM for 3 hours. The luminescence generated from the two luciferases was measured. Cycloheximide, which stalls the elongation phase (Schneider-Poetsch et al., 2010), inhibited the translation of Renilla and firefly luciferase reporter systems equally (Fig. 3.9). On the other hand, pateamine A which inhibits the initiation phase of translation (Low et al., 2005) inhibited only the luminescence from the firefly luciferase. The Renilla luciferase encoded by the CrPV IRES was not inhibited. Gephyronic acid also inhibited the luminescence only from the firefly luciferase similar to pateamine A. This suggests that gephyronic acid inhibits the initiation phase.

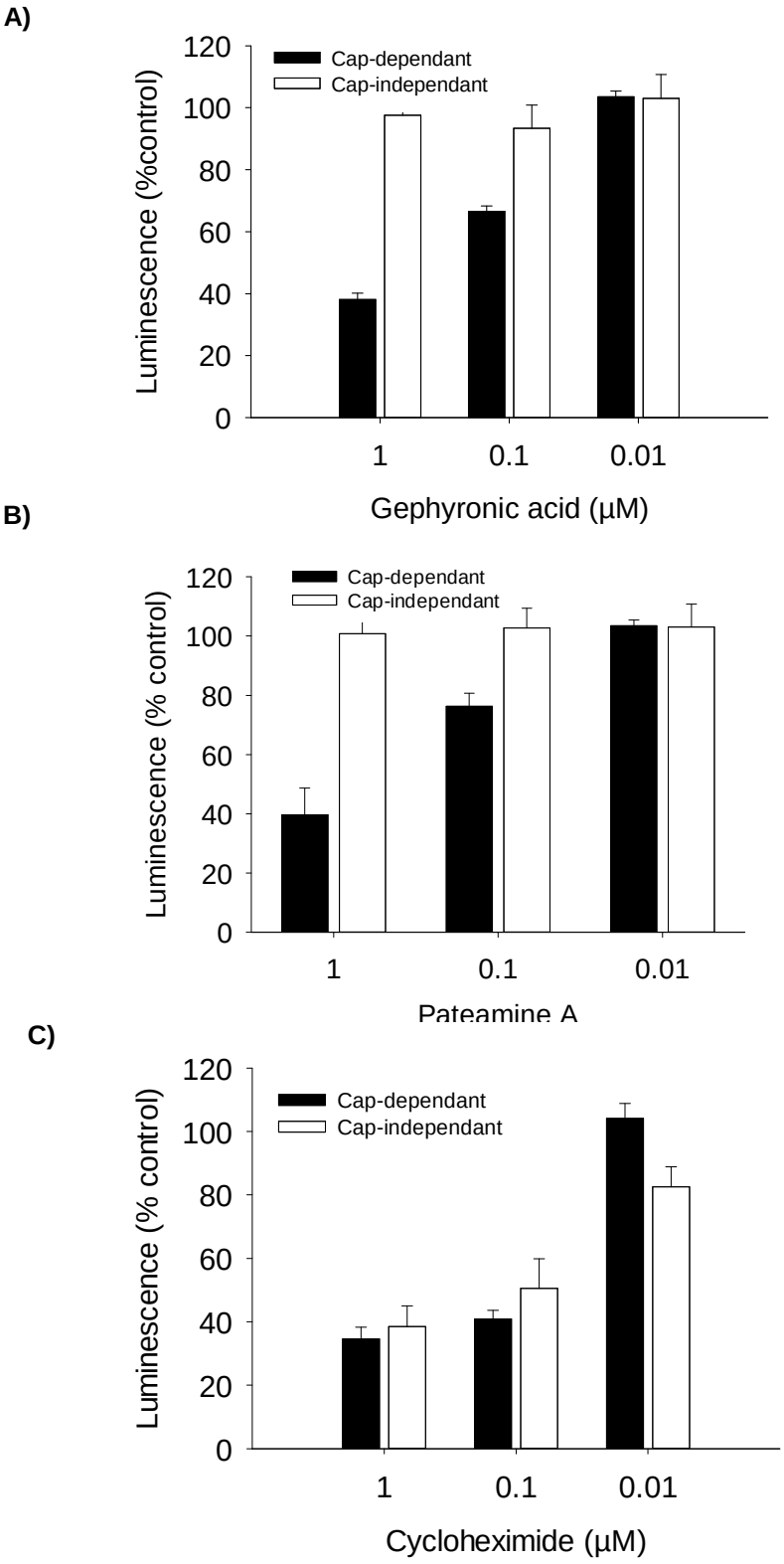



***Figure 3.9: Gephyronic acid does not inhibit CrPV IRES***

*A bicistronic vector was used to determine the phase of translation inhibition. Gephyronic acid (panel A) inhibits only the cap-dependant translation system in a similar way as pateamine A (panel B), while cycloheximide (panel C) inhibits both systems.*

### 3.2.7 Effect of gephyronic acid on the eIF4 complex

From the previous section it was clear that gephyronic acid targeted the initiation phase. To analyse if gephyronic acid targets the eIF4 complex within the initiation phase of translation a bicistronic vector consisting of firefly luciferase translated by the cap-dependant system and Renilla luciferase translation polio IRES dependant system was used (Fig. 3.8). Polio IRES does not require eIF4E and eIF4G to initiate translation as mentioned in section 1.2.6. If gephyronic acid inhibits the translation of the Renilla luciferase it suggests that gephyronic acid does not target eIf4E or eIF4G.

To determine the phase of translation inhibition induced by gephyronic acid, KB-3-1 cells were transfected with the polio IRES bicistronic vector. 48 hours after transfection the cells were treated with gephyronic acid, cycloheximide and pateamine A. Cycloheximide and patemine A were used as controls. Pateamine A inhibits translation initiation by interfering with eIF4A whereas cycloheximide inhibits the elongation phase and thus requires eIF4G and eIF4E. As a result, both pateamine A and cycloheximide inhibit the translation of the Renilla luciferase similar to the firefly luciferase (Fig 3.10). Gephyronic acid inhibits the translation of both the firefly and the Renilla luciferase equally as represented by the inhibition of the luminescence from both the luciferases. This suggests that the target of gephyronic acid does not involve eIF4G and eIF4E.

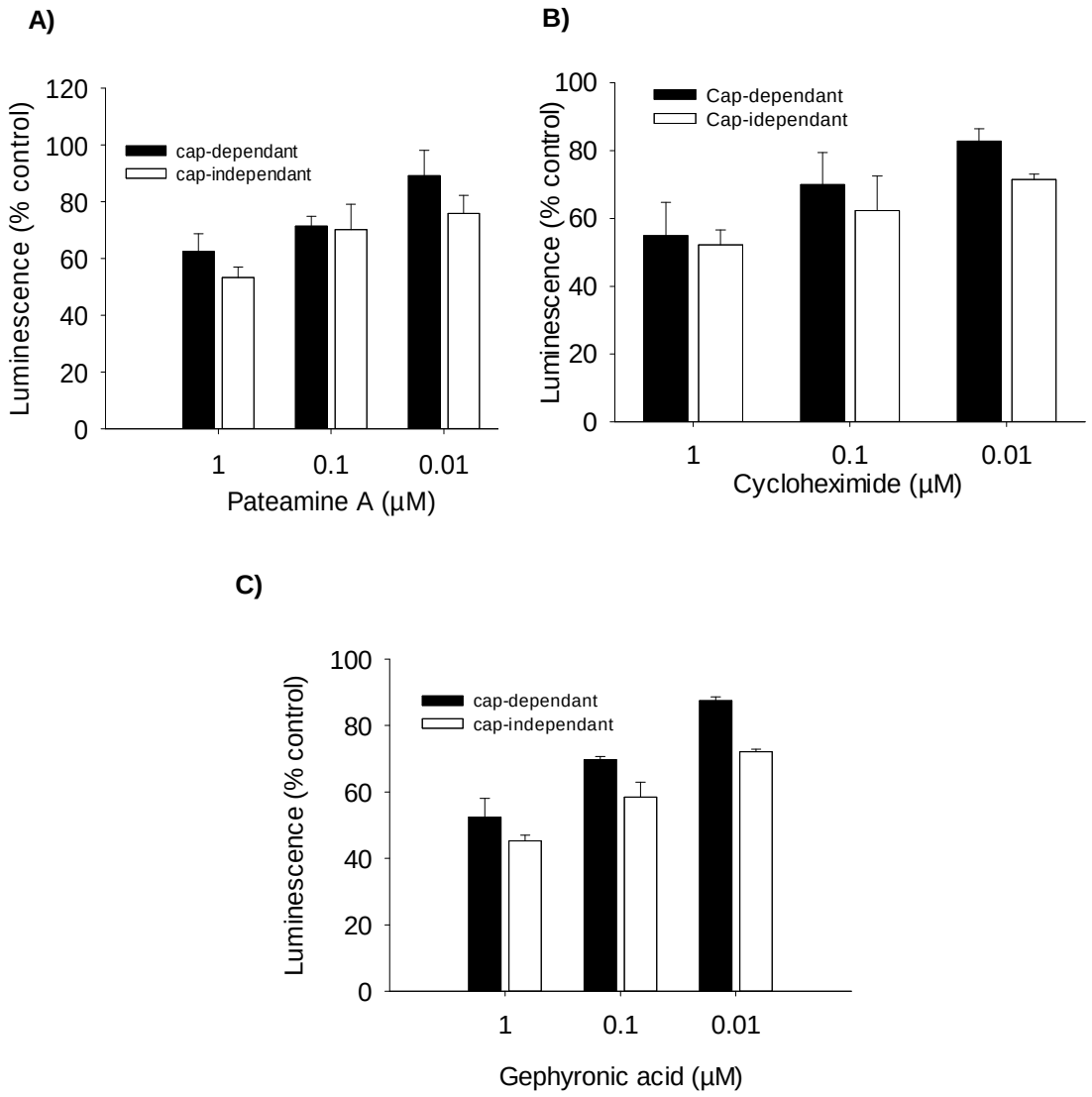



***Figure 3.10: Gephyronic acid inhibits the translation by polio IRES***

*Gephyronic acid similar to pateamine A and cycloheximide, inhibits the polio IRES translation (A, white bars) and the cap-dependant translation system.*

### 3.2.8 Elucidating the target of gephyronic acid

With the knowledge that gephyronic acid inhibits translation initiation, a fitness based chemo-genetics approach with *Saccharomyces cerevisiae* mutants was used to identify the possible target. The yeast mutants were obtained from EUROSCARF (Giaever et al., 2002; Parsons et al., 2006). Viable mutants involved in translation elongation and initiation phase were used for this assay. Treating these mutants with gephyronic acid would show an increased sensitivity when gephyronic acid binds to a target that is closely related to the knocked out pathway in the mutant. The screening results with gephyronic acid showed that most of the mutants had the same sensitivity as the wild type. As seen from Table 3.3 only the eIF2B mutant was clearly more sensitive. This suggests that the target of gephyronic acid lies in the eIF2 complex.

***Table 3.3: Chemical-genetics with yeast mutants***

| Mutant name | Gene knocked out | Inhibition zone (Diameter in mm) |
|---|---|---|
| YJL138C | eIF4A | 20 |
| YGL049C | eIF4G | 19 |
| YKL204W | eIF4E associated ptn | 20 |
| YKL081W | EF-1gamma | 20 |
| YKR026C | eIF2B alpha | 28 |
| YLR199C | Protein involved in 20S proteasome assembly | 15 |
| YAL035W | eIF5B | 20 |
| WT | - | 21 |

### 3.2.9 Elucidating the target of gephyronic acid with DARTS approach.

To identify the direct target of gephyronic acid a drug affinity responsive target stability (DARTS) approach was used. The basis of this method is that binding of a ligand to a protein target reduces the susceptibility of the protein to protease digestion due to a stabilization of the protein's folded state (Lomenick et al., 2009). KB-3-1 cell lysates were incubated with various concentrations of gephyronic acid for 2 hours and subjected to pronase digestion for 30 minutes at 37°C. Cycloheximide treated cell lysate was used as a control. Cell lysate treated with only methanol and then subjected to digestion were used as a control. The digestion was stopped by heating with SDS loading for ten minutes at 96ºC. An SDS PAGE was performed with the samples. The protein bands were stained with Coomassie Brilliant Blue. Protection of a band at ~36 kDa was noticed (Fig. 3.11.) This protection was concentration dependent. The protein from the eIF2 complex with a molecular weight of 36 KDa is eIF2 alpha.

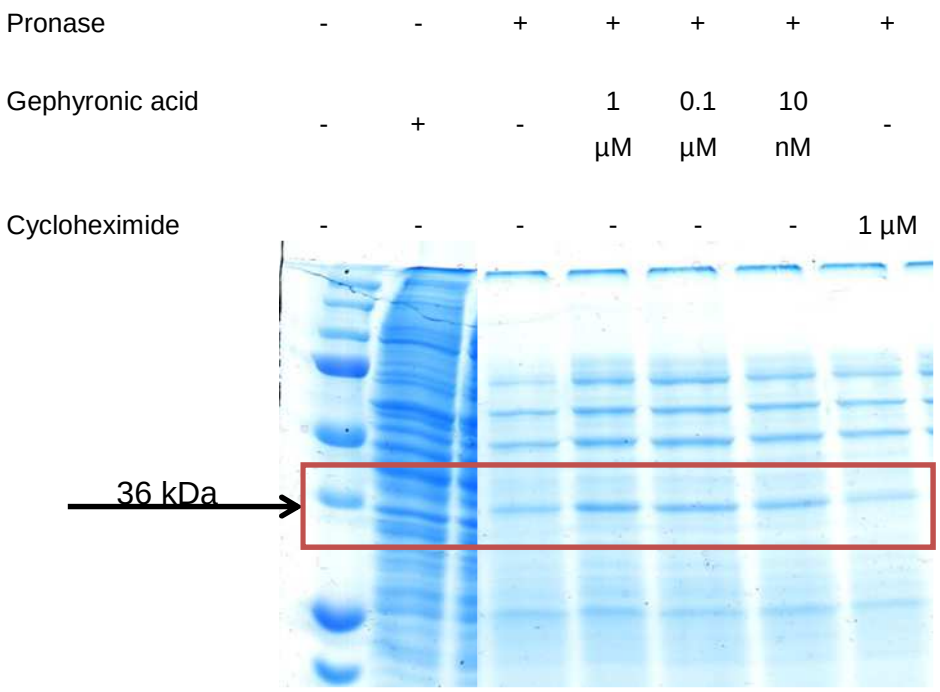



***Figure 3.11: DARTS approach with gephyronic acid***

*KB-3-1 cell lysates treated with varying concentration of gephyronic acid were subjected to a DARTS protocol with pronase. After SDS-PAGE the gel was stained with Coomasse Brilliant Blue. Lysate treated with cycloheximide served as negative control. Gephyronic acid protected a protein band at 36 kDa in a dose dependent manner. Cycloheximide did not.*

Hence the experiment was repeated with the same controls as above followed by a western blot using an anti eIF2α antibody. The blot was also re-probed with an anti-GAPDH antibody to serve as a loading control for the proteins. As can be seen from Fig. 3.12, gephyronic acid treated cells showed a concentration dependent protection of eIF2α. Cycloheximide failed to protect eIF2α from pronase digestion even at its highest concentration (250 µM).

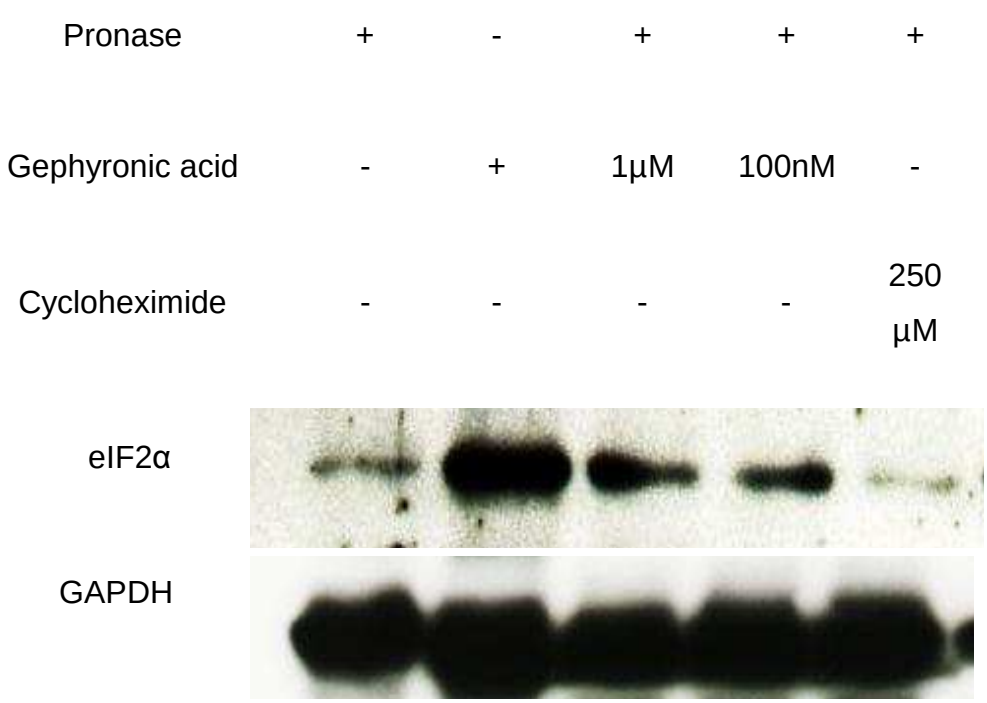



***Figure 3.12: DARTS approach with gephyronic acid***

*KB-3-1 cell lysates treated with varying concentration of gephyronic acid were subjected to digestion with pronase. Lysates treated with cycloheximide served as negative control. The lysates were then subjected to western blot with an anti-eIF2α antibody. Gephyronic acid protected eIF2α in a dose dependant manner. GAPDH was used as a loading control.*

### 3.2.10 Target fishing with biotinylated gephyronic acid

In order to fish the target protein biotinylated gephyronic acid was used with a biotin attached to the hydroxyl group at C3 (Fig. 3.13).

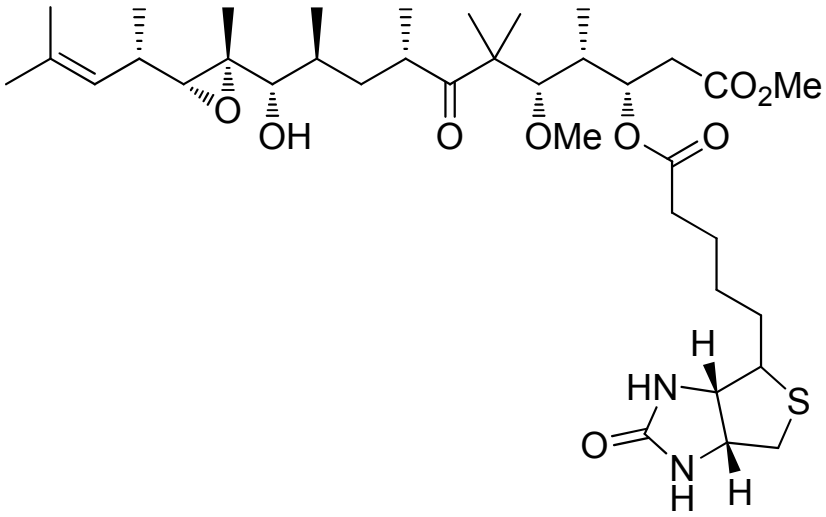



***Figure 3.13: Structure of biotinylated gephyronic acid.***

To test if the biotinylated gephyronic acid is as active as the parent compound, KB-3-1 cells were treated with biotinylated gephyronic acid for 5 days and an MTT assay was performed to assess the viability (Fig. 3.14A). The $IC_{50}$ of the compound was 45 nM, i.e. it is almost as effective as gephyronic acid. To test that the biotinylation did not alter the properties of gephyronic acid, biotinylated gephyronic acid was also tested for its translation inhibition. As seen from Fig. 3.14B, the biotinylated gephyronic acid inhibited translation almost to the same degree as gephyronic acid. The $IC_{50}$ was 0.4 µM.

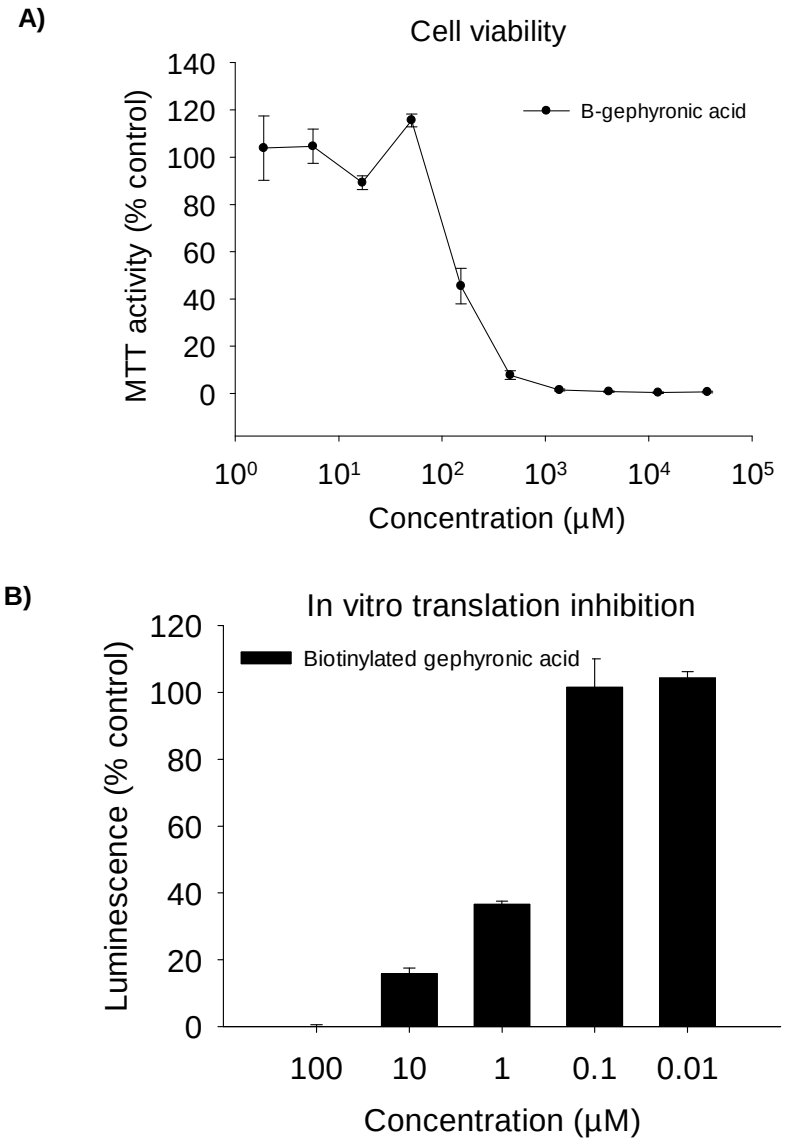



***Figure 3.14: Biotinylated gephyronic acid has parental properties***

*Biotinylated gephyronic acid was almost as cytotoxic as the parent compound with an $IC_{50}$ of 45 nM (a). Biotinylated gephyronic acid incubated with rabbit reticulocyte lysate supplemented with mRNA and amino acids inhibited translation (b).*

### 3.2.11 Immunofluorescence investigations with biotinylated gephyronic acid

To confirm the target of gephyronic acid, PtK2 cells were treated with biotinylated gephyronic acid for 3 hours. Then they were stained with Alexa Fluor 488 labelled streptavidin and an anti eIF2α antibody from mouse which was made visible by a. secondary anti mouse Alexa Fluor 594 antibody. As a result, biotinylated gephyronic acid was stained green, as was the cellular biotin, and eIF2α was stained red. Gephyronic acid treated cells served as control. Cells treated with gephyronic acid

also showed green fluorescence due to the affinity of streptavidin to mitochondrial biotin, but in cells treated with biotinylated gephyronic acid there is co-localisation of eIF2α and biotin (Fig. 3.15) whereas in the cells treated with gephyronic acid there is no co-localisation.

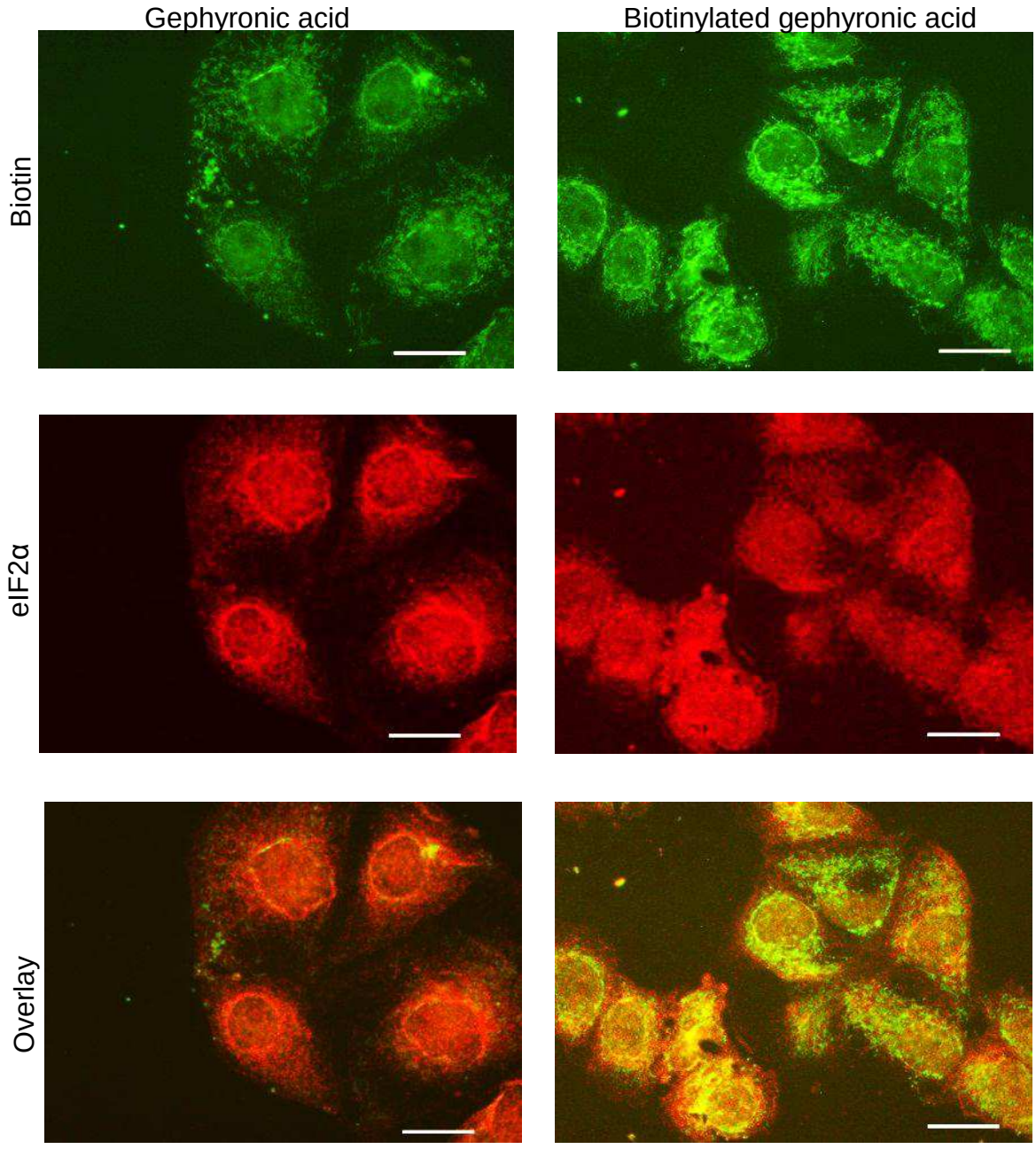



***Figure 3.15: Co-localisation studies with biotinylated gephyronic acid***

*PtK2 cells were treated with 50 nM of gephyronic acid or biotinylated gephyronic acid for 3 hours and stained using streptavidin (labelled green), which binds to biotin, and an anti-eIF2α antibody (red). Biotinylated gephyronic acid co-localises with eIF2α. Scale bar: 50 µm.*

### 3.2.12 Pull down assay with biotinylated gephyronic acid

To prove further that eIF2α is the target of gephyronic acid, a pull down assay was performed with the biotinylated gephyronic acid. A KB-3-1 cell lysate was treated with biotinylated gephyronic acid. A lysate treated with gephyronic acid served as negative control. One batch of the lysates was incubated with biotinylated gephyronic acid for one hour and then with gephyronic acid for the next hours to show competitive binding. Then the lysates were incubated with streptavidin magnetic beads. The beads were washed to remove unspecifically bound proteins and heated with 4X SDS denaturing loading dye at 96ºC for ten minutes. Proteins were separated by SDS PAGE. The gel was then subjected to silver staining. In line with previous results, a band at 36 kDa was observed in the lanes of lysates treated with the biotinylated gephyronic acid and a fainter one in the lysate that were treated with both compounds (Fig. 3.16).

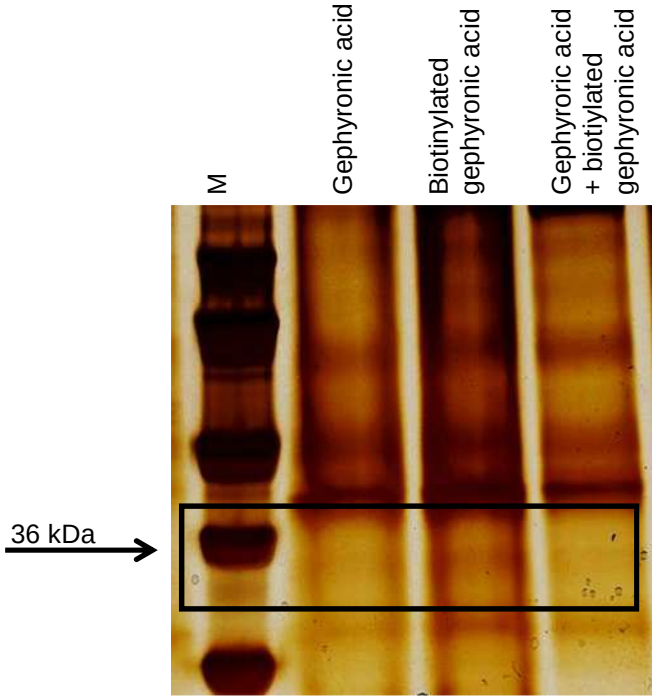



***Figure 3.16: Pull down assay with biotinylated gephyronic acid.***

*KB-3-1 cell lysates were incubated with methanol (lane 1), gephyronic acid (lane 2), biotinylated gephyronic acid (lane 3) or a mixture of biotinylated gephyronic acid and native gephyronic acid (lane 4) and the targets pulled down using streptavidin beads. The eluates were analysed by SDS-PAGE followed by silver staining. The lysate treated with biotinylated gephyronic acid or the mixture showed an additional band at 36 kDa.*

The same experimental setup was repeated and the gel was subjected to western blot using an anti eIF2α antibody. As can be seen from Fig. 3.17, lane 2 (treated with biotinylated gephyronic acid) and lane 3 (treated with biotinylated gephyronic acid and gephyronic acid) show a band whereas it is almost absent from the lane 1 and 4 which was treated with methanol or with gephyronic acid. The membrane was also probed with anti-pyruvate carboxylase (PC) antibody which is present in the mitochondria. PC is a biotin containing protein which also binds to streptavidin and is also fished. Thus, PC could serve as a loading control for the assay.

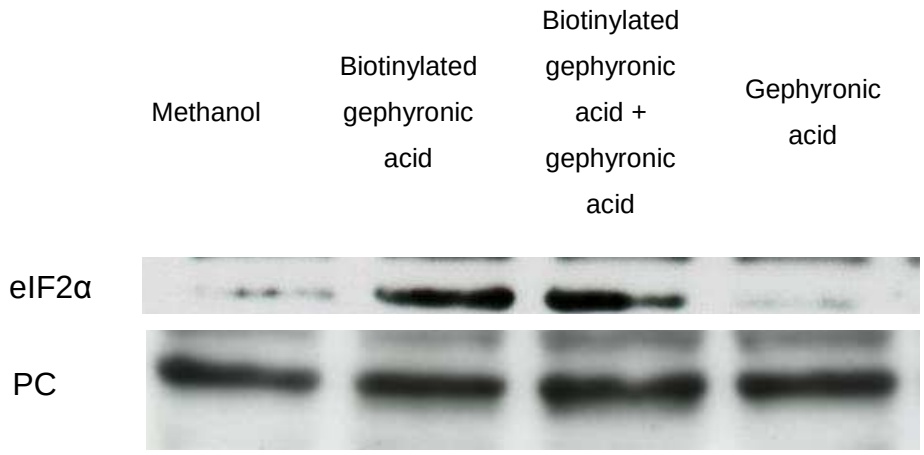



***Figure 3.17: Western blot with pulled down lysates***

*KB-3-1 cell lysates were incubated with methanol (lane 1), biotinylated gephyronic acid (lane 2), a mixture of biotinylated gephyronic acid and gephyronic acid (lane 3) or gephyronic acid (lane 4). The targets were pulled down using a streptavidin column. The eluates were analysed by SDS-PAGE followed by western blotting with anti-eIF2α antibody. PC served as loading control. The lysate treated with biotinylated gephyronic acid and the mixture showed a band of eIF2α whereas the lysates treated with methanol or gephyronic acid did not show a band. PC is equally present in all lanes (MW = 129 kDa).*

### 3.2.13 Predicting the binding pocket of gephyronic acid in eIF2α

To locate the binding pocket of gephyronic acid in eIF2α, docking calculations were performed using the Vina program. Since the crystal structure of the target region for eIF2α specific kinases is known, the coordinates deposited in the Protein Data Bank with the accession number 1Q46 could be used for the calculations (Dhaliwal and Hoffman, 2003). An inactive isomer of gephyronic acid (synthetic) reported by Anderl et al. (2011) was also used for the docking calculations (Fig. 3.18)

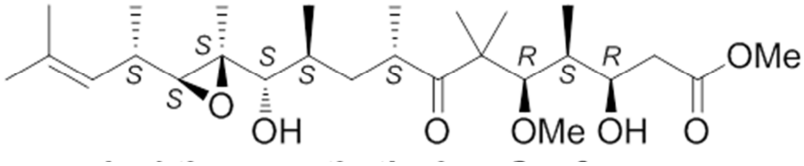



***Figure 3.18: Structure of gephyronic acid isomer used for docking calculations***

The PODRG server was used to get the coordinates and charges (Schuttelkopf and van Aalten, 2004). The known 3D chiral centres were checked. For the inactive isomer the chiral centres were inverted using Jmol and the charges reassigned on the PRODRG server. During a first run the residues involved in the interaction were determined. In a second run torsion angle changes in all related side chains were allowed. The calculations were performed using the known 3D structure of gephyronic acid and the related stereo isomer. The results showed that the aliphatic ends of gephyronic acid and its inactive isomer were bound to Ala 130 and Ala 140, the carboxylic ends to Glu 15 and Asp 17. The natural gephyronic acid was additionally bound to Tyr 39, which increases the binding energy from 26 to 29 kJ/mol. As seen in Fig. 3.19, this is obviously due to the geometry related to the epoxide group.

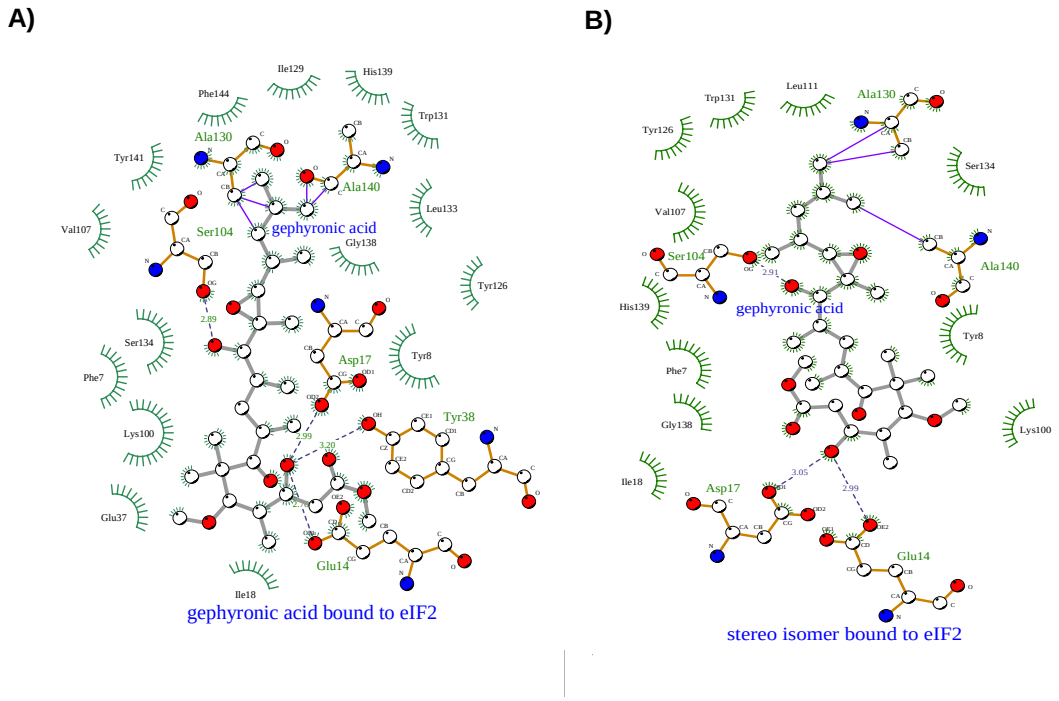



***Figure 3.19: The binding pocket of gephyronic acid***

*(**a**) The calculated binding pocket of gephyronic acid , (**b**) the stereo isomer shows much lower affinity.*

## 3.3 Identification of mode action of myriaporone 3/4

As described in Sections 3.1.3 and 3.1.4 myriaporone 3/4 inhibited translation both *in vitro* and in cells in nanomolar ranges, but the mechanism of translation inhibition and the target protein had still to be elucidated.

### 3.3.1 Analysis of myriaporone cytotoxicity by FACS

To test the kinetics of cell death induction by myriaporone 3/4, A-431 cells were treated with 90 nM of the polyketide for 3, 6, and 24 hours. The induction of apoptosis in the cells was measured by flow cytometry (FACS). Methanol treated cells were used as control. From Fig. 3.20 it can be seen that within 6 hours of treatment with myriaporone 3/4, a significant percentage of cells (14 %) underwent apoptosis.

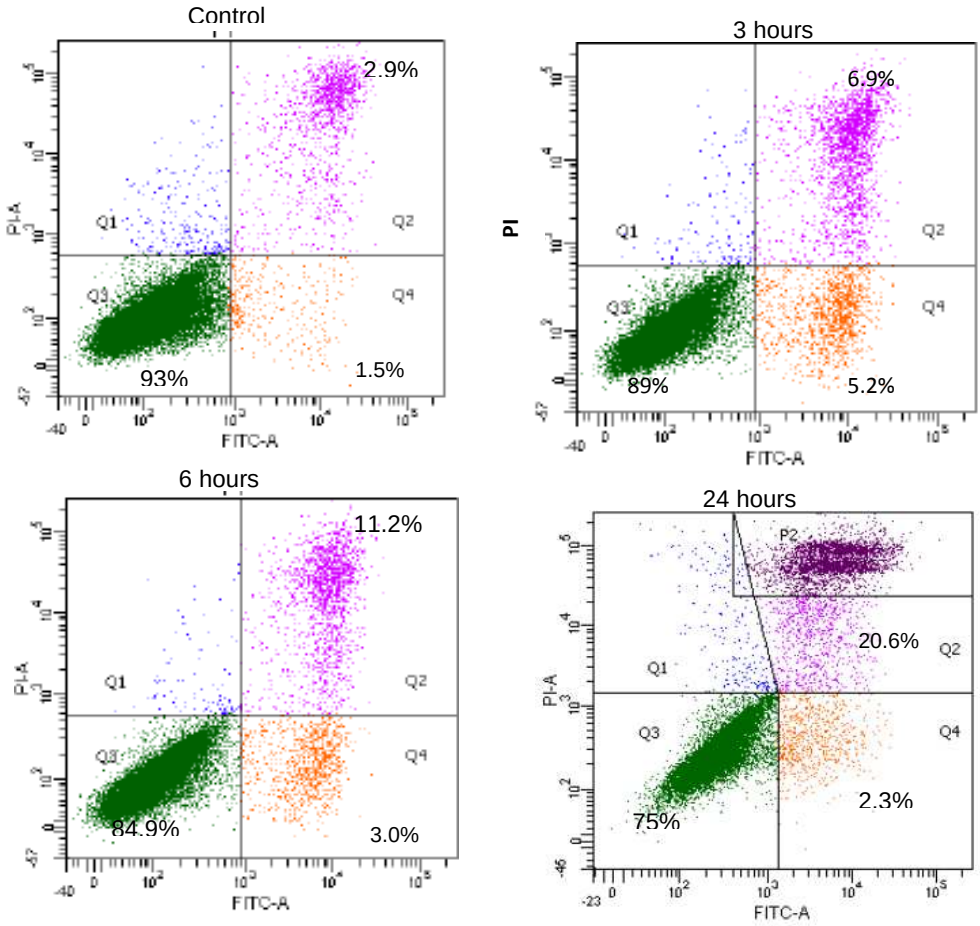



***Figure 3.20: FACS analysis of apoptotic effects in A-431 cells***

*Myriaporone 3/4 induced a time dependant apoptosis as can be seen by the increasing numbers of cells in quadrant 4 and 2.*

### 3.3.2 Activity of myriaporone 3/4 in primary cells

Though myriaporone 3/4 was active in nanomolar ranges in the cancerous cell lines, the effect could be less pronounced in primary cells. Therefore cells of the tumour cell line KB-3-1 and primary fibroblasts (NHDF) were incubated with increasing concentrations of myriaporone 3/4 for 48 and 72 hours. As a parameter of viability, the cells were assayed for their ATP content. While the viability of KB-3-1 cells was strongly reduced resulting in an $IC_{50}$ of 0.2 and 0.6 µM after 48 and 72 hours, respectively, the NHDF cells were much less affected. Even after 72 hours the highest concentration applied (20 µM) showed only 20 % reduction in cell viability (Fig. 3.21). As a result the primary cells were around 300 times less sensitive to myriaporone 3/4 than the cancer cells.

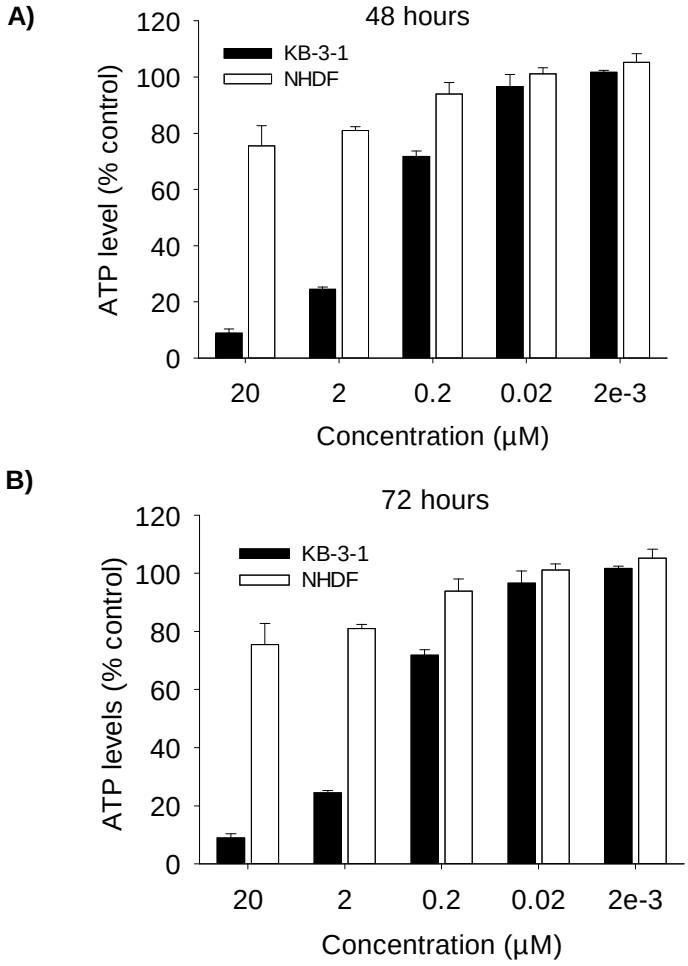



***Figure 3.21: Cytotoxicity of myriaporone 3/4 to primary and cancer cells***

*Myriaporone 3/4 showed less cytotoxic effect to primary NHDF cells (white bars) compared to cancerous KB-3-1 cells (black bars) after an incubation of 48 (A) and 72 hours (B).*

### 3.3.3 Influence of myriaporone 3/4 on angiogenesis

As mentioned earlier translation inhibitors have been shown to inhibit angiogenesis. To test if myriaporone could inhibit angiogenesis, an *in vitro* tube formation model was used. HUVECs plated in Matrigel® were treated with myriaporone 3/4 in a concentration range of 2.0 – 0.002 µM. Myriaporone inhibited the tube formation with an MIC of 0.04 µM.

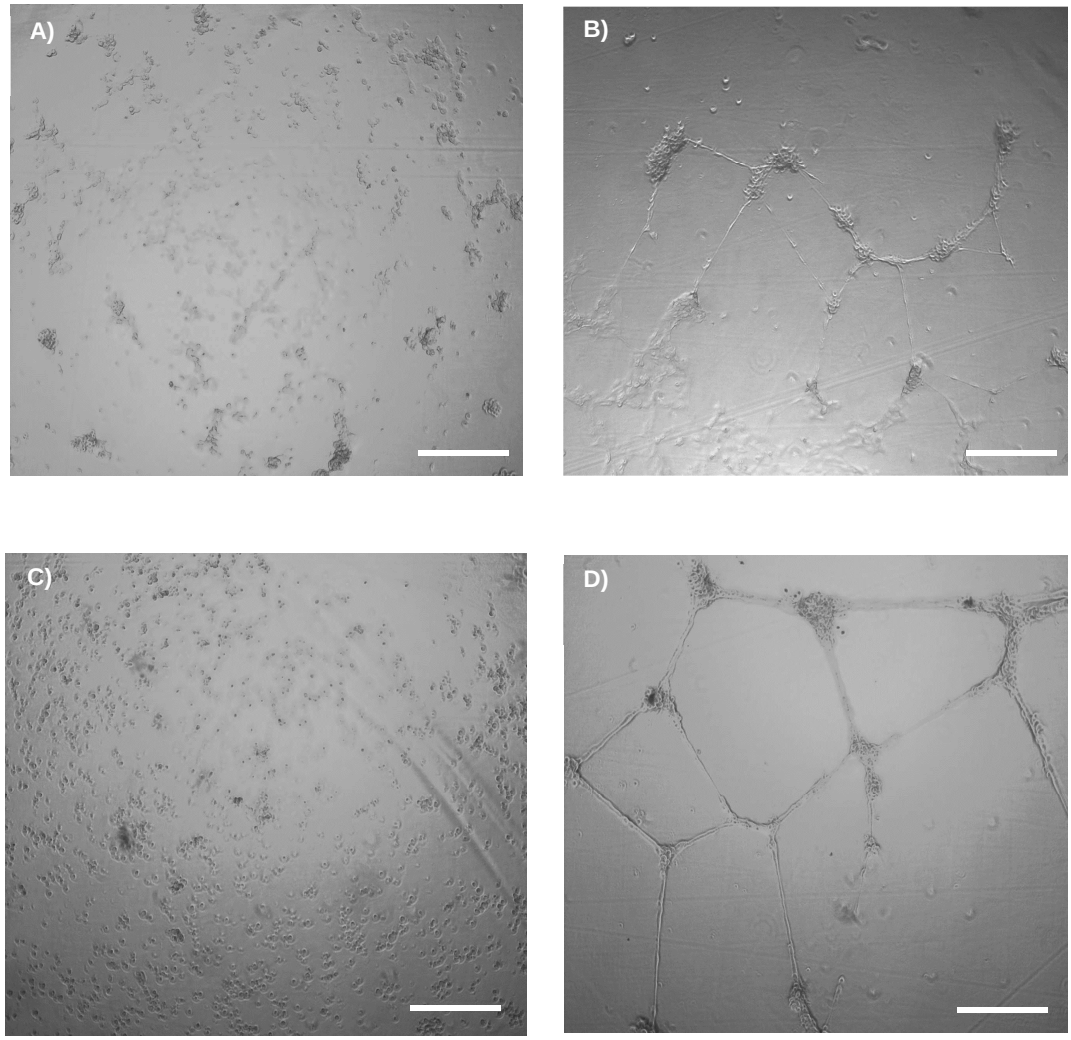



***Figure 3.22: Myriaporone 3/4 inhibits tube formation by HUVECs***

*The cells were treated with various concentrations of myriaporone. Panel A (2 µM of myriaporone 3/4) and panel B (80 nM of myriaporone 3/4) are representative figures. Panel C shows HUVECs treated with 200 nM suramin as a positive control. Cells in panel D were treated with the vehicle methanol only. Scale bar: 50 µm.*

### 3.3.4 Comparison of the effects of myriaporone 3/4 on eukaryotic and prokaryotic translation

To analyse if myriaporone 3/4 is a general eukaryotic translation inhibitor, a wheat germ lysate supplemented with luciferase mRNA and amino acids was employed. The mixture treated with various concentration of myriaporone 3/4 was incubated at 37ºC for 60 minutes and the luminescence was measured. Myriaporone 3/4 inhibited the translation in the wheat germ lysate (Fig. 3.23 A) as already seen with the reticulocyte lysate. As myriaporone 3/4 was inactive in prokaryotes as seen in Section 3.1. It should not inhibit the prokaryotic translation system. To test this, *E. coli* cell lysate was supplemented with luciferase mRNA and amino acid and incubated with different concentrations of myriaporone 3/4 at 30ºC. The resulting luminescence was measured after two hours. As expected myriaporone 3/4 did not inhibit the translation of the *E. coli* system (Fig. 3.23 B).

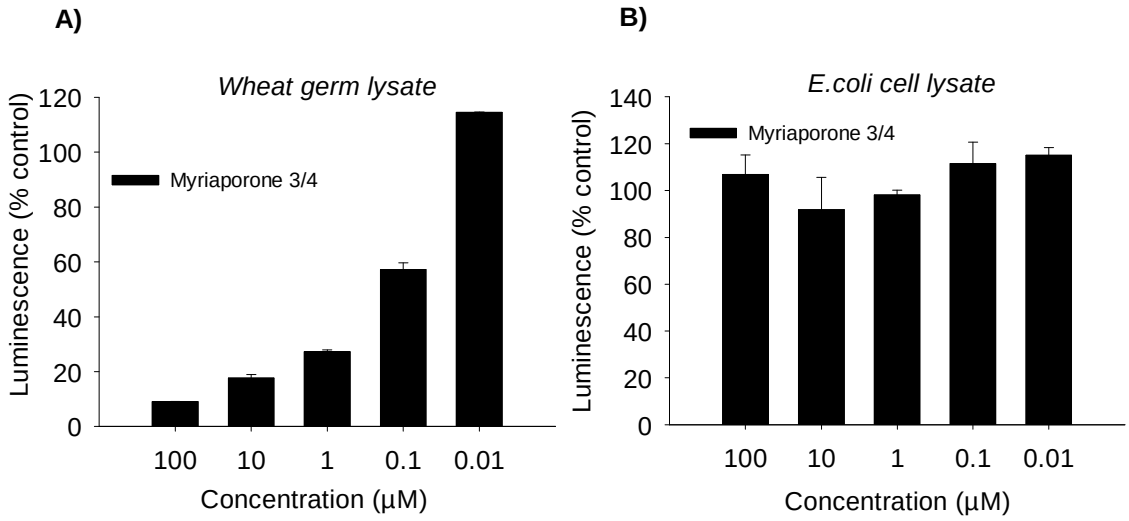



***Figure 3.23: Myriaporone 3/4 does not inhibit prokaryotic translation***

*Wheat germ lysates (A) and E. coli cell lysates (B) were incubated with luciferase mRNA, amino acids and myriaporone 3/4 in various concentrations. The luminescence plotted with respect to control shows a dose dependent reduction in translation only in the wheat germ lysate.*

### 3.3.5 Effect of myriaporone 3/4 on translation initiation

From the translation inhibition assays it was seen that myriaporone 3/4 inhibited translation. To determine if myriaporone 3/4 disrupts translation initiation the bicistronic system as mentioned in Section 3.2.6 which consisted of firefly luciferase translated by a cap-dependant and Renilla luciferase translated by CrPV IRES systems was used. For the CrPV IRES all the initiation factors are dispensable. If the translation is nevertheless stopped, it implies that myriaporone 3/4 does not target the initiation phase. KB-3-1 cells transfected with the above vector were treated with myriaporone 3/4, pateamine A and cycloheximide at concentrations ranging from 1.0 - 0.01 µM for 3 hours. The luminescence generated from the two luciferases was measured. Myriaporone 3/4 inhibited the translation of Renilla and firefly luciferase from the reporter systems equally like cycloheximide. Pateamine A inhibited only the cap dependant system as it inhibits the initiation phase. From this experiment, it is clear that myriaporone is rather an inhibitor of the elongation phase.

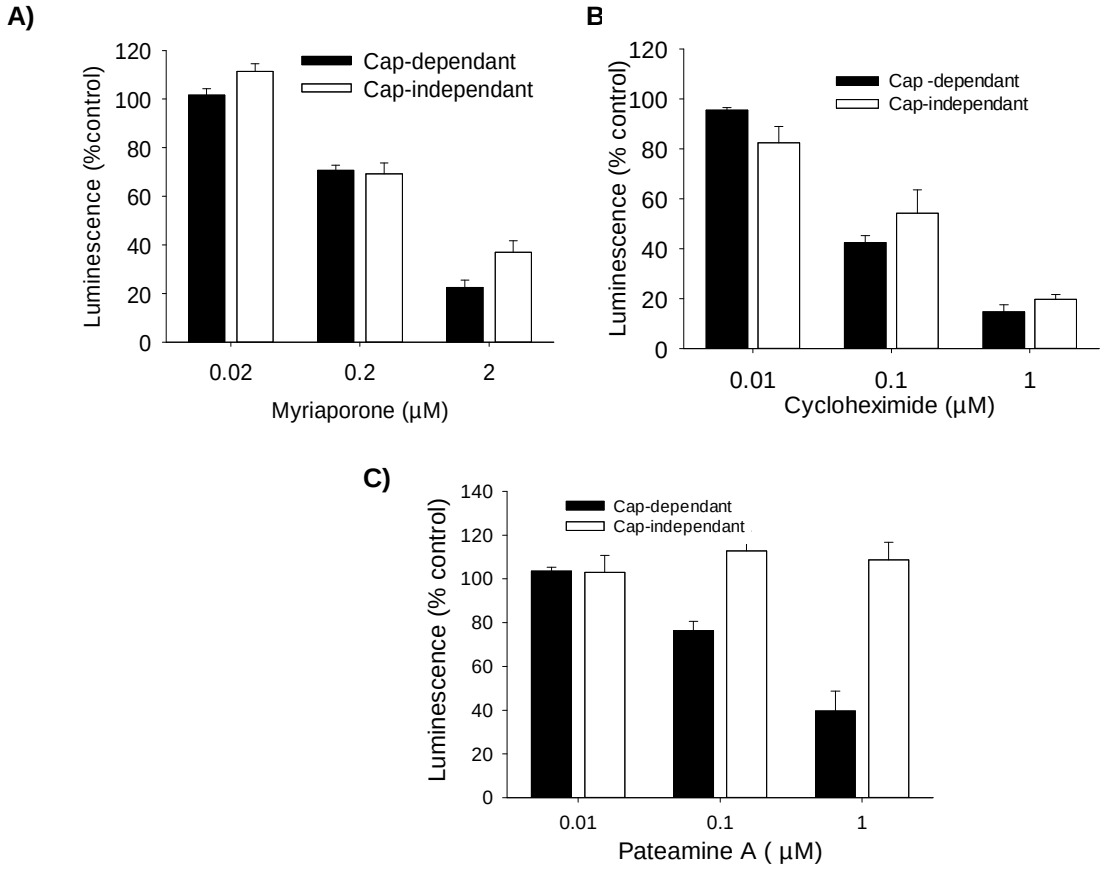



***Figure 3.24: Myriaporone inhibits CrPV IRES***

*Myriaporone 3/4 (A) like cycloheximide (B) inhibits the translation of the Renilla luciferase coded by the CrPV IRES (white bars) similar to the cap-dependant translation of the firefly luciferase (black bars). Pateamine A which targets the initiation inhibits only the cap-dependant translation.*

### 3.3.6 Influence of myriaporone 3/4 on eIF4 complex

Though myriaporone 3/4 inhibited translation of CrPV IRES, it could have a target within the eIF4 complex. To determine if myriaporone 3/4 disrupts the eIF4 complex, the bicistronic system as mentioned in Section 3.2.6 which consisted of firefly luciferase translated by a cap-dependant system and Renilla luciferase translated by polio IRES systems was used. For the polio IRES only eIF4G and eIF4E are dispensable. If the translation is nevertheless stopped, it cannot be due to interference with the eIF4 complex. KB 3-1 cells transfected with the above vectors were treated with myriaporone 3/4, pateamine A and cycloheximide at concentrations ranging from 1 0 - 0.01 µM for 3 hours. The luminescence generated from the two luciferases was measured. Myriaporone 3/4 like cycloheximide and pateamine A (targets eIF4A) inhibited the translation of Renilla and firefly luciferase from the reporter systems equally. This suggests that myriaporone does not target eIF4E or eIF4G.

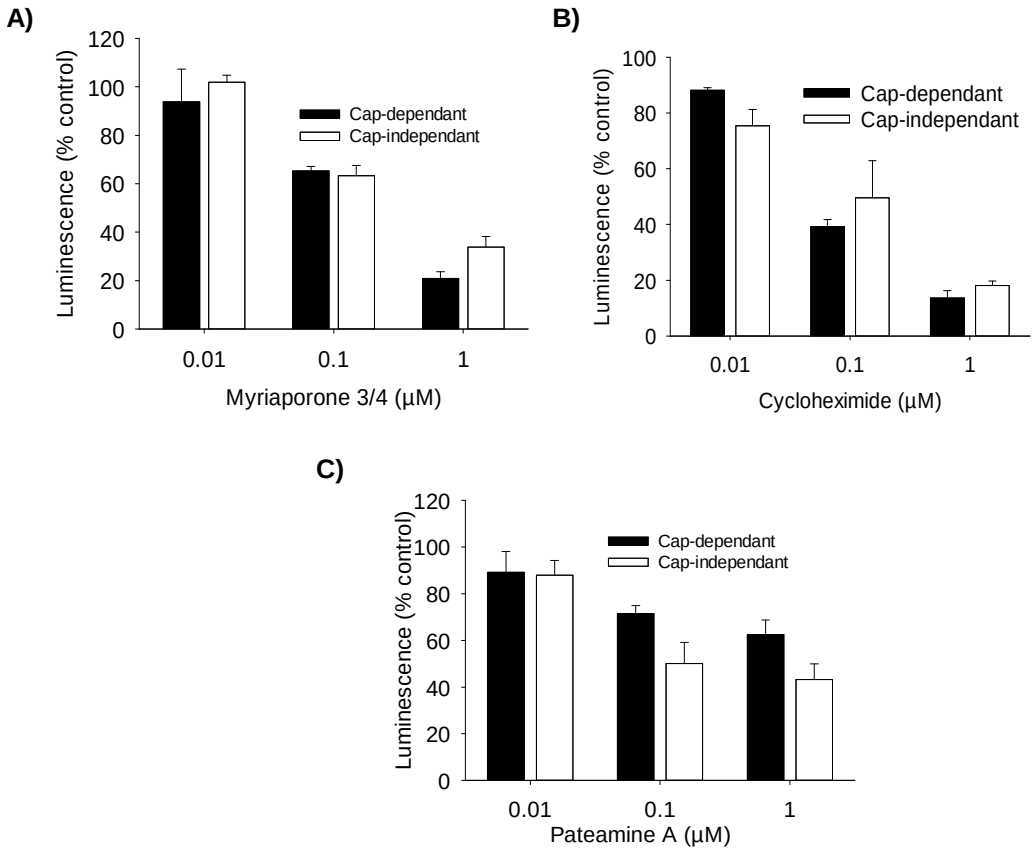



***Figure 3.25: Myriaporone 3/4 inhibits polio IRES dependant translation***

*Myriaporone 3/4 (A) like cycloheximide (B) and pateamine A (C) inhibits the translation of the Renilla luciferase coded by the polio IRES (white bars) similar to the cap-dependant translation of the firefly luciferase (black bars).*

### 3.3.7 Chemo-genetic interaction screening with myriaporone 3/4

Since myriaporone 3/4 did not target the initiation phase it was hypothesised that it inhibits translation in the elongation phase. To test this further, a fitness based chemo-genetics approach with yeast mutants was used. Viable knock out mutants of genes involved in translation elongation and initiation phase were used. The chemical-genetic interaction was tested using an agar diffusion assay. Paper discs soaked with 20 µg of myriaporone 3/4 were placed on YPD agar plates, which were inoculated with the mutant of interest. As expected, mutants with relation to initiation factors did not show an increased sensitivity. The elongation factor eEF1γ mutant also failed to show any change in the sensitivity compared to the WT which suggested that eEF1 was also not influenced by myriaporone 3/4 (Table 3.4).

***Table 3.4: Results of a chemo-genetic interaction screening with yeast mutants***

| Mutant name | Gene knocked out | Inhibition zone (mm) |
|---|---|---|
| **YJL138C** | eIF4A | 15 |
| **YGL049C** | eIF4G | 15 |
| **YKL204W** | eIF4E associated ptn | 14 |
| **YKL081W** | EF-1gamma | 13 |
| **YKR026C** | eIF2B alpha | 14 |
| **YLR199C** | Ptn involved in 20S proteasome assembly | 15 |
| **YAL035W** | eIF5B | 17 |
| **WT** | - | 15 |

### 3.3.8 Influence of myriaporone 3/4 on the phosphorylation of eEF2

Myriaporone 3/4 did not affect any of the mutants in the yeast screening. Also from Section 3.3.5 it is clear that myriaporone does not target the initiation phase. Therefore it was assumed that myriaporone 3/4 could target the elongation phase. The elongation phase regulation is achieved by altering the phosphorylation state of eEF2. However, the eEF2 mutant is not viable in yeast. So the phosphorylation state of eEF2 was tested in mammalian cells. KB-3-1 cells cultured on coverslips in a four well plate were treated with 50 nM of myriaporone 3/4 for 4 hours. Cells treated with methanol served as control. The cells were then fixed and stained with anti-phosphorylated-eEF2 antibody. As can be seen from the Fig. 3.26, cells treated with myriaporone 3/4 show increased levels of phosphorylation as indicated by the increased green fluorescence.

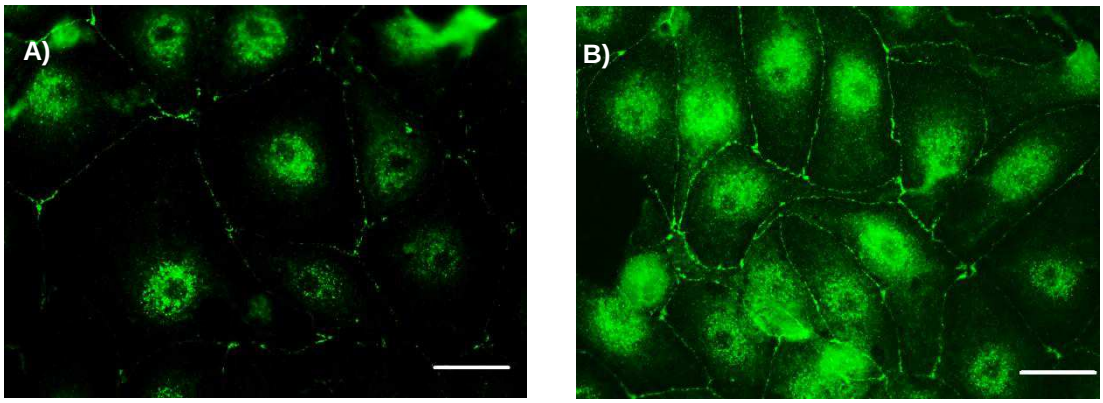



***Figure 3.26: Myriaporone 3/4 induces phosphorylation of eEF2***

*KB-3-1 cells were treated with either 50 nM of myriaporone 3/4 (panel B) or methanol which served as control (A) for 4 hours and stained with phospho-eEF2 antibody (green). Cells treated with myriaporone 3/4 showed an increased phosphorylation compared to the control. Scale bar 50 µm.*

To reconfirm the above effect KB-3-1 cells were treated with 1 µM, 0.1 µM and 0.01µM myriaporone 3/4 for 6 hours. The cell lysate was subjected to western blot and probed with phosphorylated eEF2, total eEF2 and GAPDH. GAPDH served as a loading control. As can be seen from Fig. 3.27, cells treated with myriaporone 3/4 showed an increased level of phosphorylation when compared to the control. Thus, it is clear that myriaporone 3/4 inhibits translation by inducing phosphorylation of eEF2.

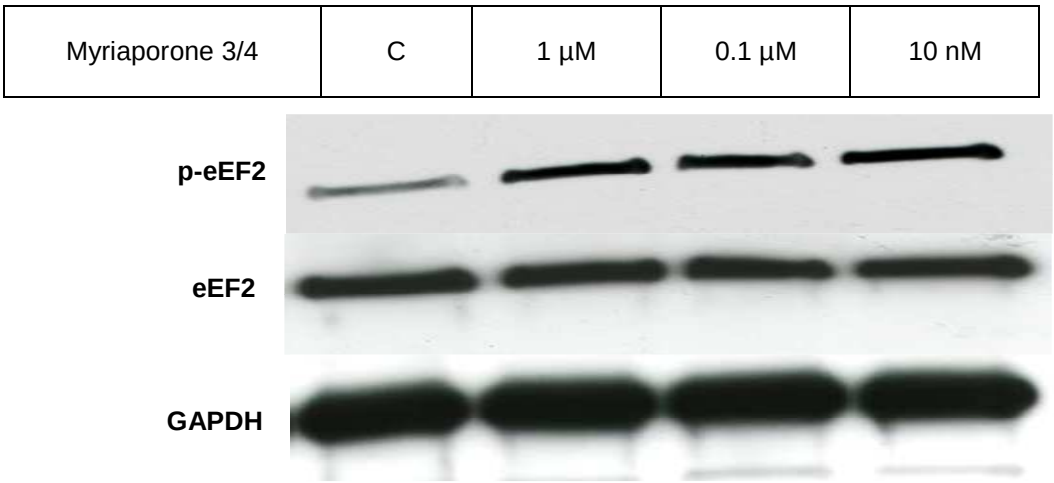



***Figure 3.27: Myriaporone 3/4 induces phosphorylation of eEF2***

*KB-3-1 cells treated with myriaporone 3/4 at various concentrations for 4 hours show an increase in the phophorylation of eEF2 compared to methanol treated cells. There is no change in the total eEF2 in treated cells compared to the control cells.*

### 3.3.9 Effect of myriaporone 3/4 on eEF2K

As seen in the previous chapter (Section 3.3.8) myriaporone induces phosphorylation of eEF2. The kinase known to phosphorylate eEF2 is eEF2K. For eEF2K to be active it has to be de-phosphorylated at Ser 366. Therefore, lysates from KB-3-1 cells treated with 1 µM, 0.1 µM, and 0.01 µM of myriaporone 3/4 were subjected to western blot and probed with anti-phosphorylated eEF2K and anti-total eEF2k antibodies. As can be seen from Fig. 3.28, myriaporone 3/4 did not alter the phosphorylation of eEF2K.

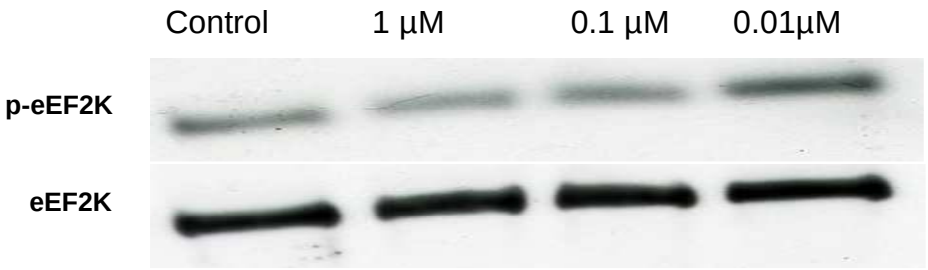



***Figure 3.28: Myriaporone 3/4 does not induce eEF2K de-phosphorylation***

*KB-3-1 cells treated with myriaporone 3/4 at various concentrations for 4 hours did not show a difference in phosphorylation when compared to cells treated with methanol (control).*

### 3.3.10 Target of myriaporone 3/4

Since myriaporone 3/4 does not alter the phosphorylation state of eEF2K, it was hypothesised that myriaporone could bind to eEF2K and thus activate. To investigate this hypothesis a DARTS approach was carried out with cell lysates treated with myriaporone 3/4 at different concentrations (1 µM, 0.1 µM, 0.01 µM). A cell lysate treated with methanol was used as negative control and a cell lysate treated with myriaporone but not subjected to pronase digestion was used as positive control. It is clear from the Fig. 3.29 that eEF2K of cell lysates treated with myriaporone were significantly protected from pronase digestion in comparison to the positive control whereas the eEF2K cell lysates treated with methanol only were not protected and almost completely digested by pronase. The membrane was also probed with anti-eEF2 antibody to check if myriaporone 3/4 binds to eEF2. It can be seen from Fig. 3.29 that eEF2 was not protected by pronase digestion as it was digested to the same extent as the negative control.

| Pronase | + | - | + | + | + |
|---|---|---|---|---|---|
| Myriaporone 3/4 | - | + | + | + | + |
| µM | 0 | 1 | 1 | 0.1 | 0.01 |

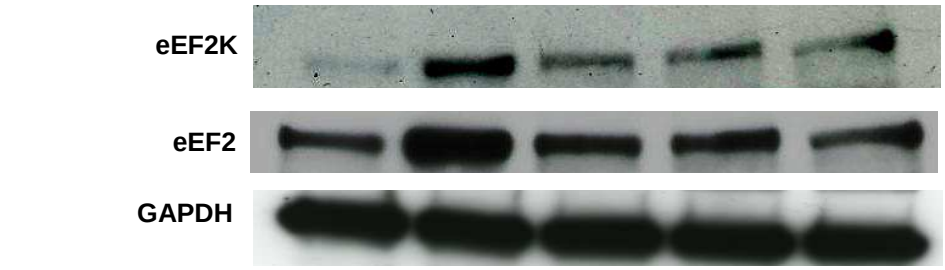



***Figure 3.29: Myriaporone binds to eEF2K directly.***

*KB-3-1 cell lysates treated with myriaporone 3/4 or methanol was subjected to pronase digestion. Western blot was performed with the lysates. Myriaporone 3/4 protected eEF2K from pronase digestion in a dose dependant manner, whereas the methanol treated lysates were digested completely. eEF2 on the other hand was not protected by myriaporone 3/4.*

To reconfirm that myriaporone 3/4 has a direct effect on eEF2K, rabbit reticulocyte lysate was supplemented with an eEF2K enriched lysate or an empty vector lysate from HEK2 cells. An *in vitro* translation assay was performed by adding luciferase mRNA and various concentrations of myriaporone 3/4 (0.01 to 1000 µM) to the above enriched lysates. The reaction mixture supplemented with an eEF2K enriched cell

lysate had an $IC_{50}$ of 80 µM and the reaction mixture enriched with the empty vector had an $IC_{50}$ of 3 µM (Fig. 3.30). These results also confirmed that the target of myriaporone is eEF2K.

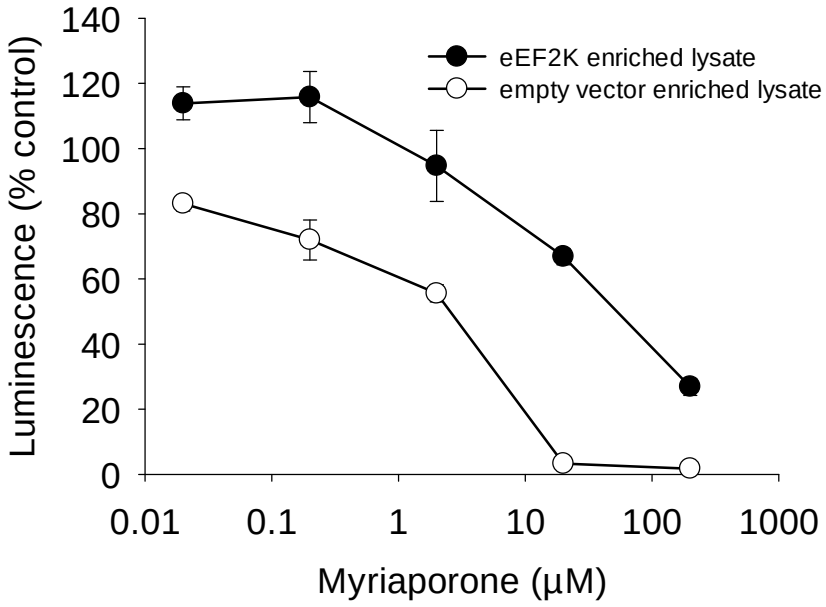



***Figure 3.30: Effect of myriaporone on eEF2K enriched lysates***

*Rabbit reticulocyte lysates were incubated with an eEF2K overexpression cell lysate or a cell lysate with an empty vector and varying concentration of myriaporone. The eEF2K enriched lysate had an higher $IC_{50}$ than the empty vector.*

## 3.4 Identification of mode action of des-epoxy tedanolide

In Sections 3.1.3 and 3.1.4 it has been reported that des-epoxy tedanolide inhibited translation both *in vitro* and in cells in nanomolar ranges. However, the mechanism of the inhibition and its target still had to be elucidated. The following sections deal with the mechanism of translation inhibition by des-epoxy tedanolide.

### 3.4.1 Analysis of des-epoxy tedanolide cytotoxicity by FACS

To test the kinetics of cell death induction by des-epoxy tedanolide, A-431 cells were treated with 50 nM of polyketide for 3, 6, 12 and 24 hours. The induction of apoptosis in the cells was measured by flow cytometry (FACS). Methanol treated cells were used as control. Fig. 3.31 shows that after 6 hours of treatment with des-epoxy tedanolide, 30 % of the cells had undergone apoptosis.

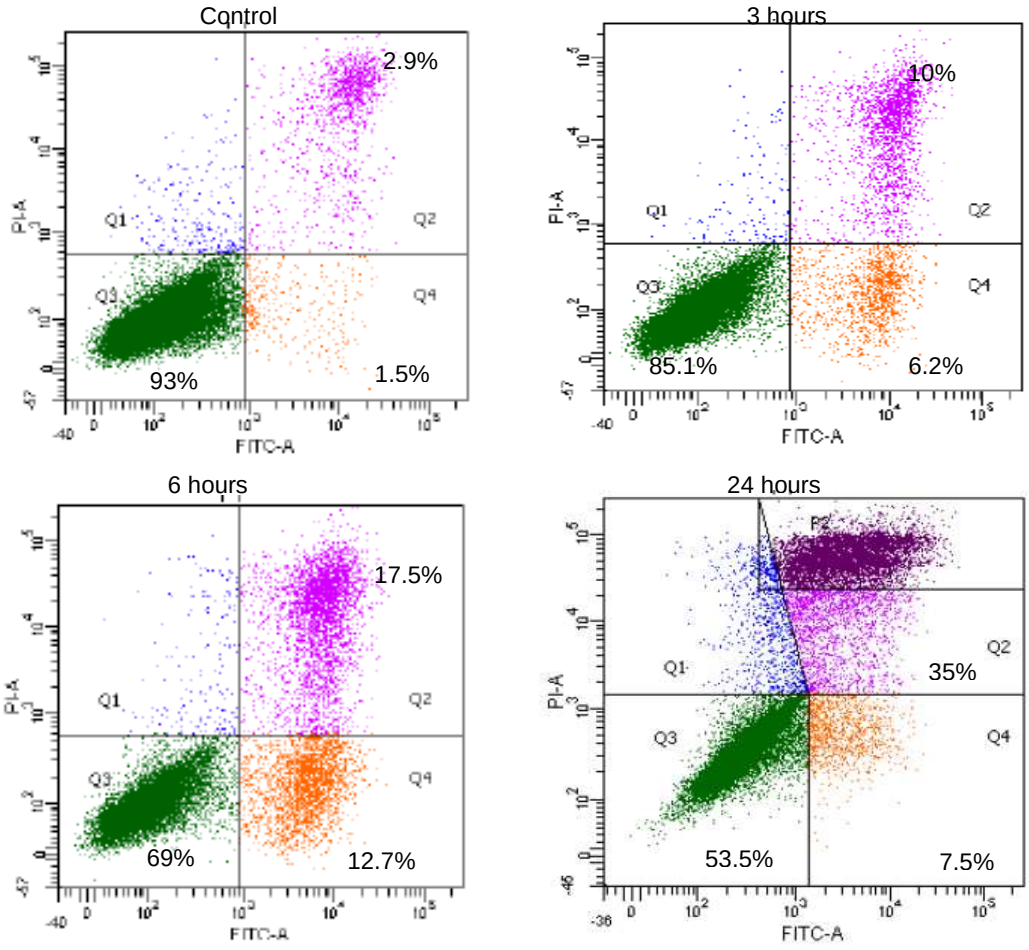



***Figure 3.31: FACS analysis of A-431 cells treated with des-epoxy tedanolide***

*42.5% of the treated cells had undergone apoptosis after 24 hours compared to 4.5 % of cells treated with methanol for 24 hours.*

### 3.4.2 Des-epoxy tedanolide inhibits angiogenesis

As mentioned earlier translation inhibitors have been shown to inhibit angiogenesis. To test des-epoxy tedanolide for such an activity, HUVECs plated in Matrigel® were treated with des-epoxy tedanolide in the concentration range of 1.0 – 0.001 µM. Des-epoxy tedanolide inhibited tube formation with an MIC of 3.6 nM.

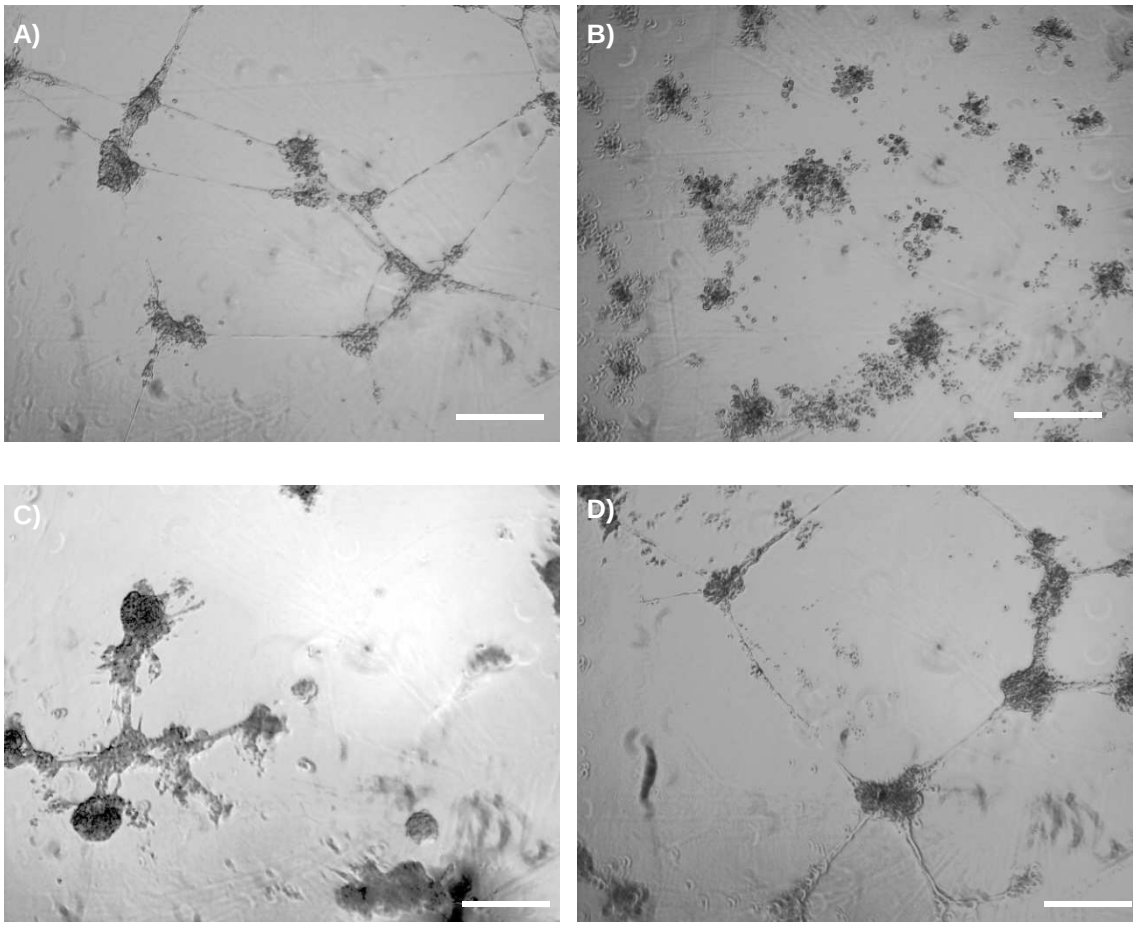



***Figure 3.32: Des-epoxy tedanolide inhibits formation of tubes in vitro***

*HUVECs plated on Matrigel were treated with various concentrations of des-epoxy tedanolide. Panel B (100 nM), panel C (3.6 nM) and panel D (0.1 nM) are representative figures. Panel A shows cells treated with the vehicle methanol only.*

### 3.4.3 Mechanism of des-epoxy tedanolide induced translation inhibition

Candidaspongiolide, a member of the tedanolide family (see Section 1.6.2), has been shown to inhibit translation by inducing phosphorylation of eIF2α. To test if des-epoxy tedanolide induces phosphorylation of eIF2α, PtK2 cells cultured on cover slips in 4-well plates were treated with 50 nM of des-epoxy tedanolide for 4 hours. The cells were fixed and stained with mouse anti-eIF2α and rabbit anti-phospho-eIF2α. Cells treated with methanol served as control. As can be seen from Fig. 3.33, cells treated with des-epoxy tedanolide showed an increased level of phosphorylation of eIF2α when compared to the control cells.

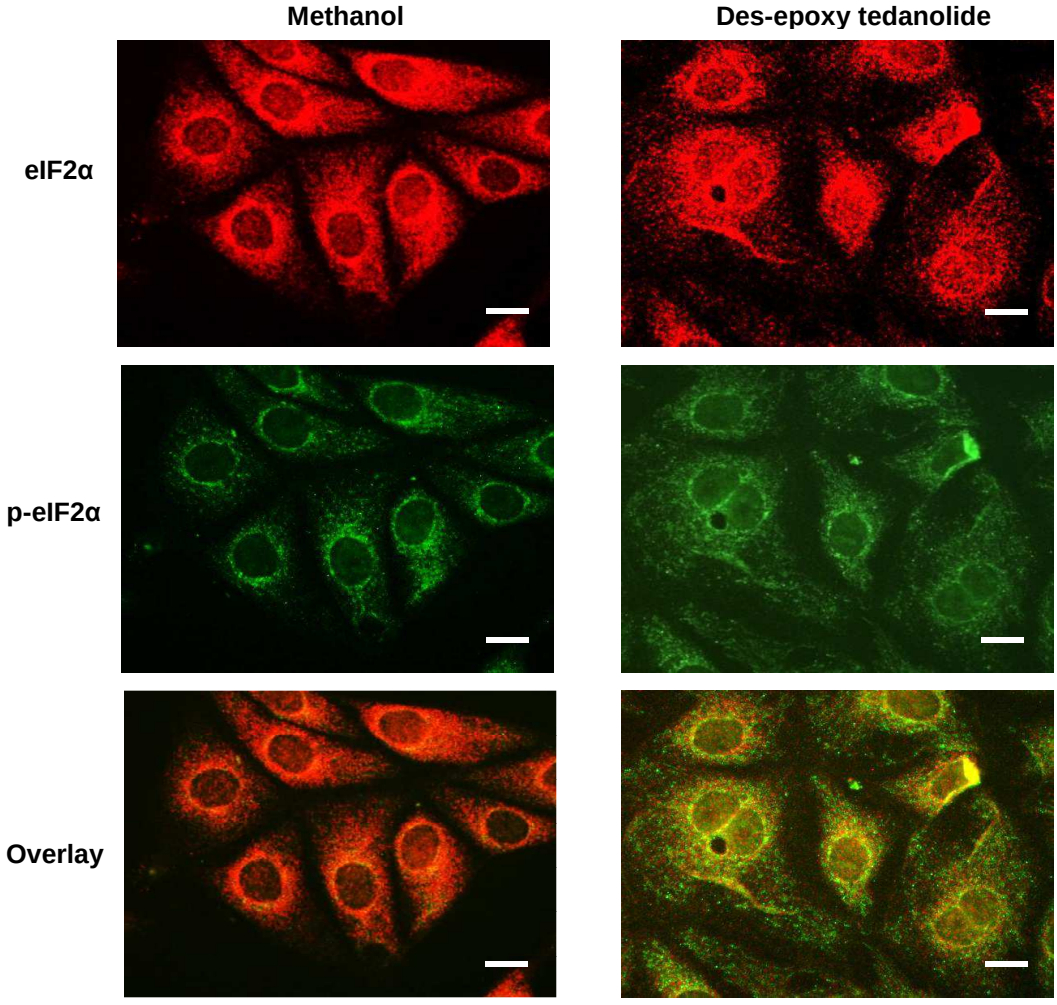



***Figure 3.33: Des-epoxy tedanolide induces phosphorylation of eIF2α***

*Cultured PtK2 cells were treated with des-epoxy tedanolide or methanol for 4 hours, fixed and stained with anti-eIF2α antibody (red) and anti-phospho-eIF2α antibody (green). Des-epoxy tedanolide induced phosphorylation of eIF2α as seen from increased green fluorescence. Scale bar: 10 µm.*

### 3.4.4 Effect of des-epoxy tedanolide on translation initiation

As mentioned earlier, CrPV IRES does not require initiation factors for the formation of the 80S initiation complex. Since des-epoxy tedanolide induces phosphorylation of eIF2α it was hypothesised that the CrPV IRES translation would not be inhibited by des-epoxy tedanolide. Therefore, KB 3-1 cells transfected with the CrPV bicistronic vector were treated with des-epoxy tedanolide, cycloheximide and pateamine A at concentrations ranging from 1 - 0.01 µM for 3 hours. The luminescence generated from the two luciferases was measured. Des-epoxy tedanolide and cycloheximide inhibited the translation of Renilla and firefly luciferase reporter systems equally (Fig. 3.34). On the other hand, pateamine A which inhibits the initiation phase of translation inhibited only the luminescence from the firefly luciferase and the Renilla luciferase encoded by the CrPV IRES was uninhibited. This suggests that des-epoxy tedanolide could have another target in the translation system apart from eIF2α.

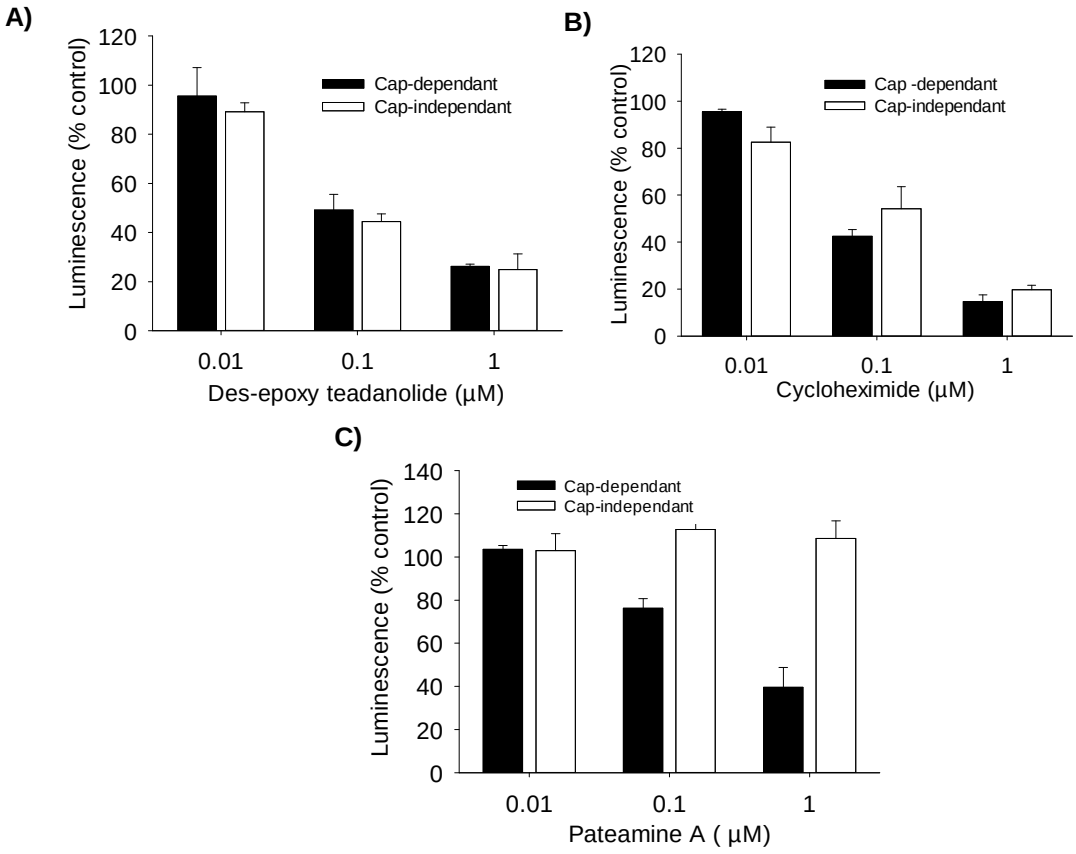



***Figure 3.34: Des-epoxy tedanolide inhibits translation initiation***

*Des-epoxy tedanolide (A) and cycloheximide (B) inhibited the translation of the Renilla luciferase coded by the CrPV IRES (white bars) similarly to the cap-dependant translation of the firefly luciferase (black bars). Pateamine A which targets eIF4A inhibited the cap-dependant translation only.*

### 3.4.5 Effect of des-epoxy tedanolide on eIF4 complex

Though des-epoxy tedanolide inhibited the CrPV IRES dependant translation, it still could target eIF4E or eIF4G. To determine if des-epoxy tedanolide targets these two factors, the bicistronic system as mentioned in Section 3.2.6 which consisted of firefly luciferase translated by a cap-dependant system and Renilla luciferase translated by polio IRES systems was used. Polio IRES does not require eIF4E and eIF4G for translation initiation. KB 3-1 cells transfected with the above vectors were treated with des-epoxy tedanolide, pateamine A and cycloheximide at concentrations ranging from 1.0 - 0.01 µM for 3 hours. The luminescence generated from the two luciferases was measured. Cycloheximide, which stalls the elongation phase, inhibited the translation of Renilla and firefly luciferase from the reporter systems equally whereas pateamine A only inhibited the firefly and not the Renilla luminescence as it inhibits eIF4A. Des-epoxy tedanolide 3/4 inhibited the translation of the polio IRES and the cap-dependent translation systems equally similarly to cycloheximide (Fig. 3.35).

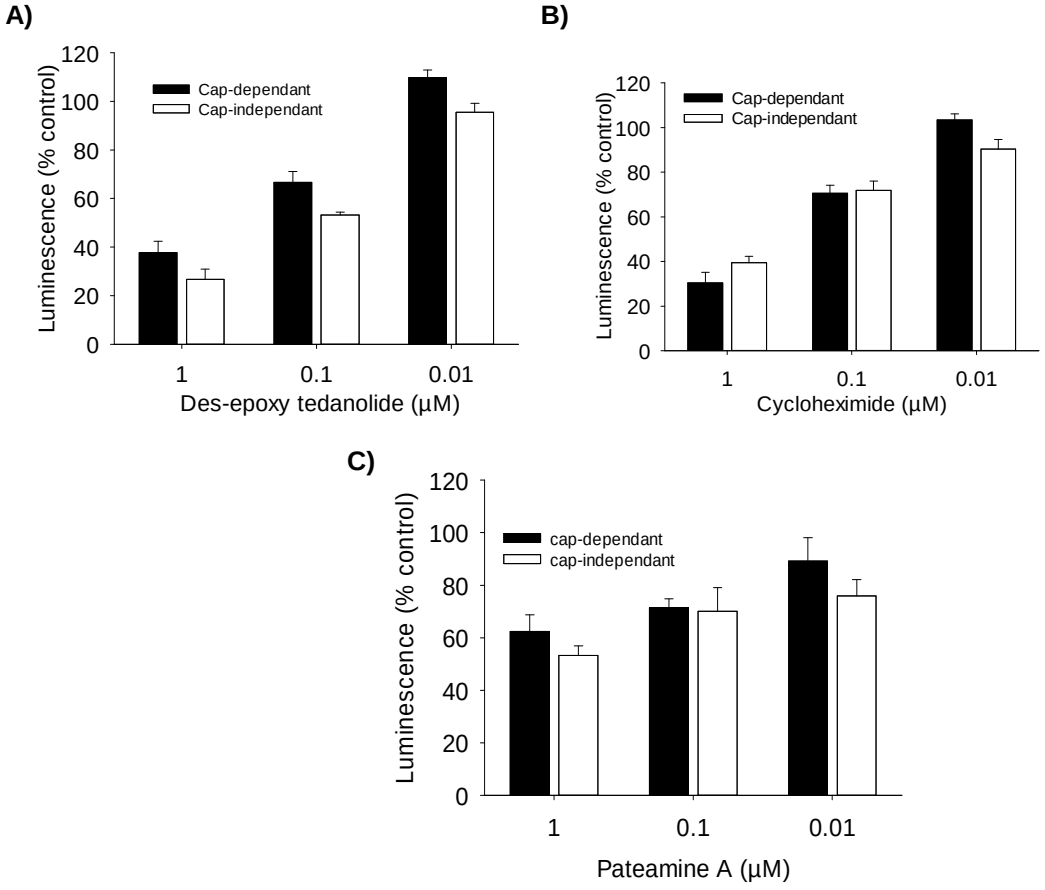



***Figure 3.35: Des-epoxy tedanolide does not disrupt eIF4 complex***

*Des-epoxy tedanolide (A) like cycloheximide (B) and pateamine A (C) inhibited the translation of the Renilla luciferase coded by the polio IRES (white bars) similarly to the cap-dependant translation of the firefly luciferase (black bars).*

### 3.4.6 Role of des-epoxy tedanolide in eEF2 inactivation

Although des-epoxy tedanolide induced the phosphorylation of eIF2α, it inhibited the translation of the CrPV IRES. This suggested that des-epoxy tedanolide could have a secondary target in the translation system. To test this hypothesis, PtK2 cells were treated with 50 nM of the polyketide and incubated for 4 hours. The cells were then fixed and stained for phosphorylated eEF2. Cells treated with methanol served as control. Des-epoxy tedanolide induced phosphorylation of eEF2 as can be seen from increased red fluorescence (Fig. 3.36).

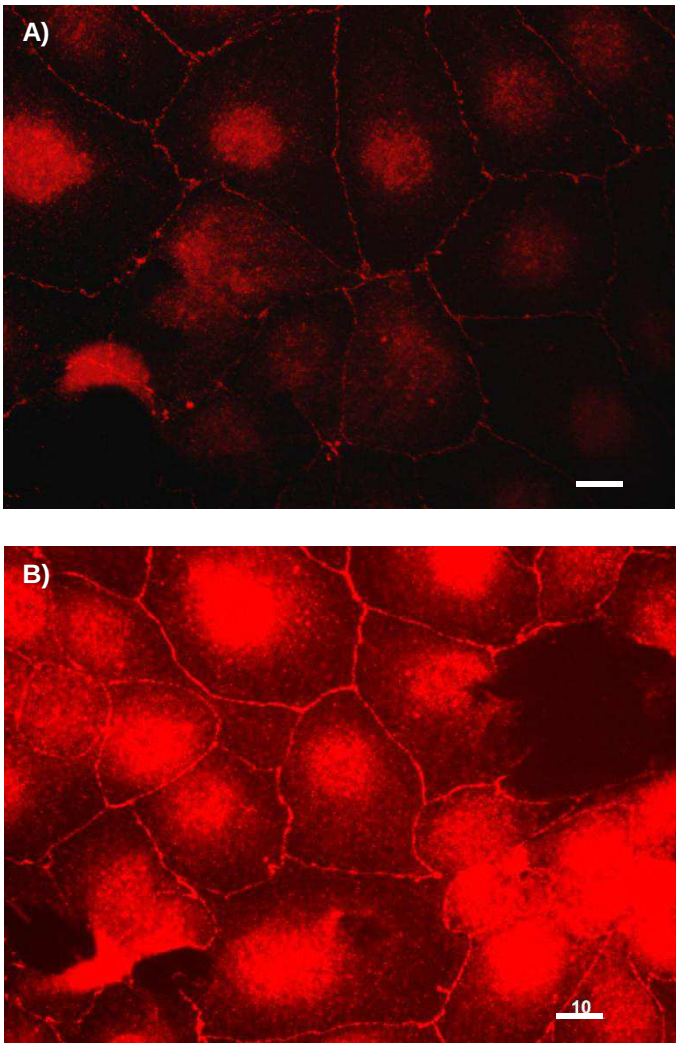



***Figure 3.36: Des-epoxy tedanolide induces phosphorylation of eEF2***

*PtK2 cells were treated either with methanol (A) or 50 nM of des-epoxy tedanolide (B) for four hours. The cells were fixed and stained for phospho-eEF2 (red). Des-epoxy tedanolide induced phosphorylation of eEF2 (A) compared to methanol treated cells (B) which served as control. Scale bar: 10 µm.*

### 3.4.7 Mechanism of eEF2 phosphorylation by des-epoxy tedanolide

As seen in the previous chapter (Section 3.4.6) des-epoxy tedanolide induced phosphorylation of eEF2. The only kinase known to phosphorylate eEF2 is eEF2K. For eEF2K to be active it has to be de-phosphorylated at Ser 366. To check if des-epoxy tedanolide reduces eEF2K phosphorylation (Ser 366), PtK2 cells were cultured on coverslips in 4-well plates for 16 hours and treated with 50 nM of des-epoxy tedanolide for 4 hours. The cells were fixed and stained for phospho-eEF2K. As can be seen from Fig. 3.37, des-epoxy tedanolide induced dephosphorylation of eEF2K.

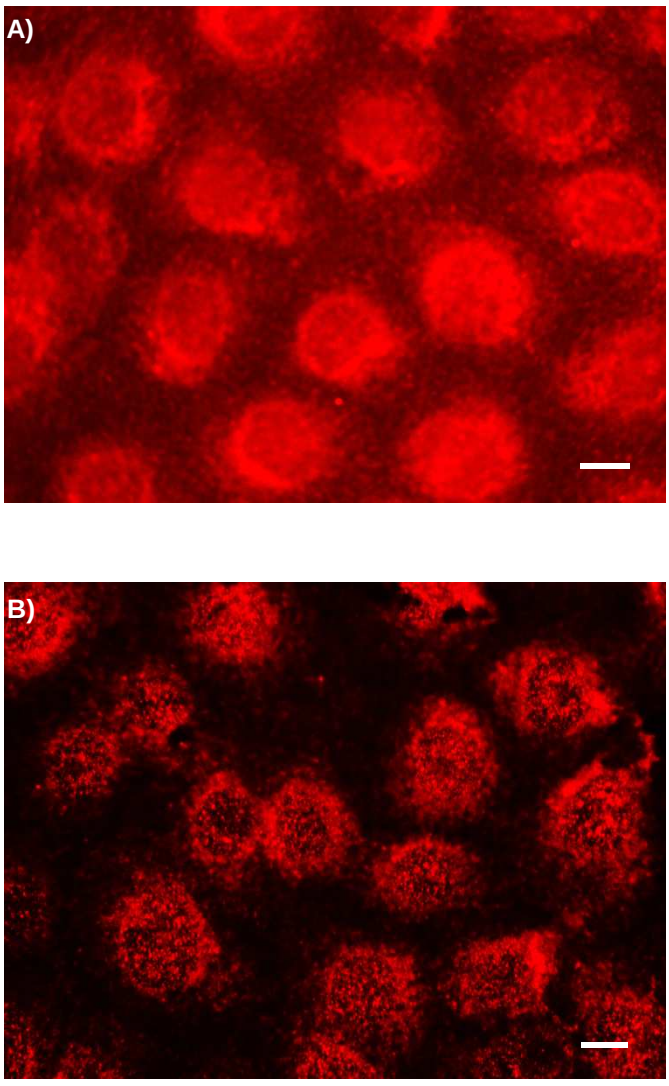



***Figure 3.37: Des-epoxy tedanolide induces dephosphorylation of eEF2K***

*PtK2 cells were treated with methanol (A) which served as control or des-epoxy tedanolide (B) for 4 hours. The cells were fixed and stained with phospho-eEF2K (Ser 366). Des-epoxy tedanolide induced dephosphorylation of eEF2K thus making it active. Scale bar: 10 µm*

## 3.5 Identification of mode action of aetheramide B

Sections 3.1.3 and 3.1.4 reported that aetheramide B inhibits translation both *in vitro* and in cells in nanomolar ranges. The following section deals with the mechanism of translation inhibition by des-epoxy tedanolide and also reports its target protein within the cell.

### 3.5.1 Aetheramide B inhibits angiogenesis

To test if aetheramide B inhibits angiogenesis, HUVECs plated in Matrigel® were treated with aetheramide B in the concentration range of 1.0 – 0.0003 µM. It was observed that aetheramide B inhibited tube formation with an MIC of 0.04 µM (Fig. 3.38).

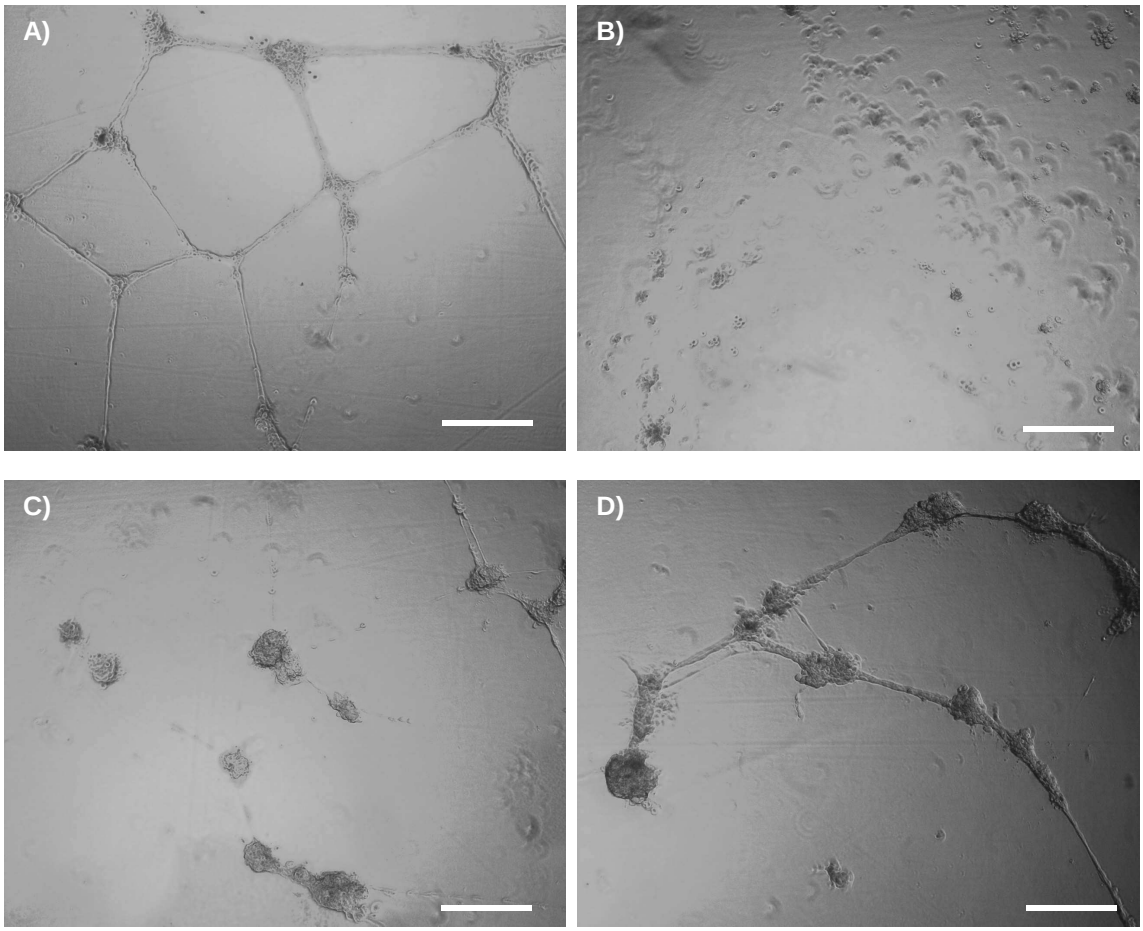



***Figure 3.38: Aetheramide B inhibits tube formation of tubes in vitro***
*HUVECs plated on Matrigel were treated with various concentrations of aetheramide B. Panel B (1.0 µM), panel C (0.04 µM) and panel D (0.1 nM) are representative figures. Panel A shows cells treated with the vehicle methanol only. Scale bar: 50 µm.*

### 3.5.2 Effect of aetheramide B on translation initiation

Since it had been shown that aetheramide B was a translation inhibitor, the next step was to identify the phase of translation which aetheramide B targets. To test if aetheramide B targets the translation initiation phase the CrPV bicistronic vector was used. As mentioned before, CrPV IRES does not require any of the initiation factors. If the translation of IRES dependant Renilla luciferase is not inhibited then it suggests that aetheramide B targets the initiation phase.

KB-3-1 cells transfected with the CrPV bicistronic vector were treated with aetheramide B, cycloheximide and pateamine A at concentrations ranging from 10 - 0.01 µM for 3 hours. The luminescence generated from the two luciferases was measured. Cycloheximide, which stalls the elongation phase, inhibited the translation of Renilla and firefly luciferase reporter systems equally (Fig. 3.39). On the other hand, pateamine A which inhibits the initiation phase of translation inhibited only the luminescence from the firefly luciferase, whereas the Renilla luciferase encoded by the CrPV IRES was uninhibited. Aetheramide B inhibited the luminescence only from the firefly luciferase. The Renilla luciferase was uninhibited similar to the results obtained with pateamine A suggesting that the target of aetheramide B lies in the initiation phase.

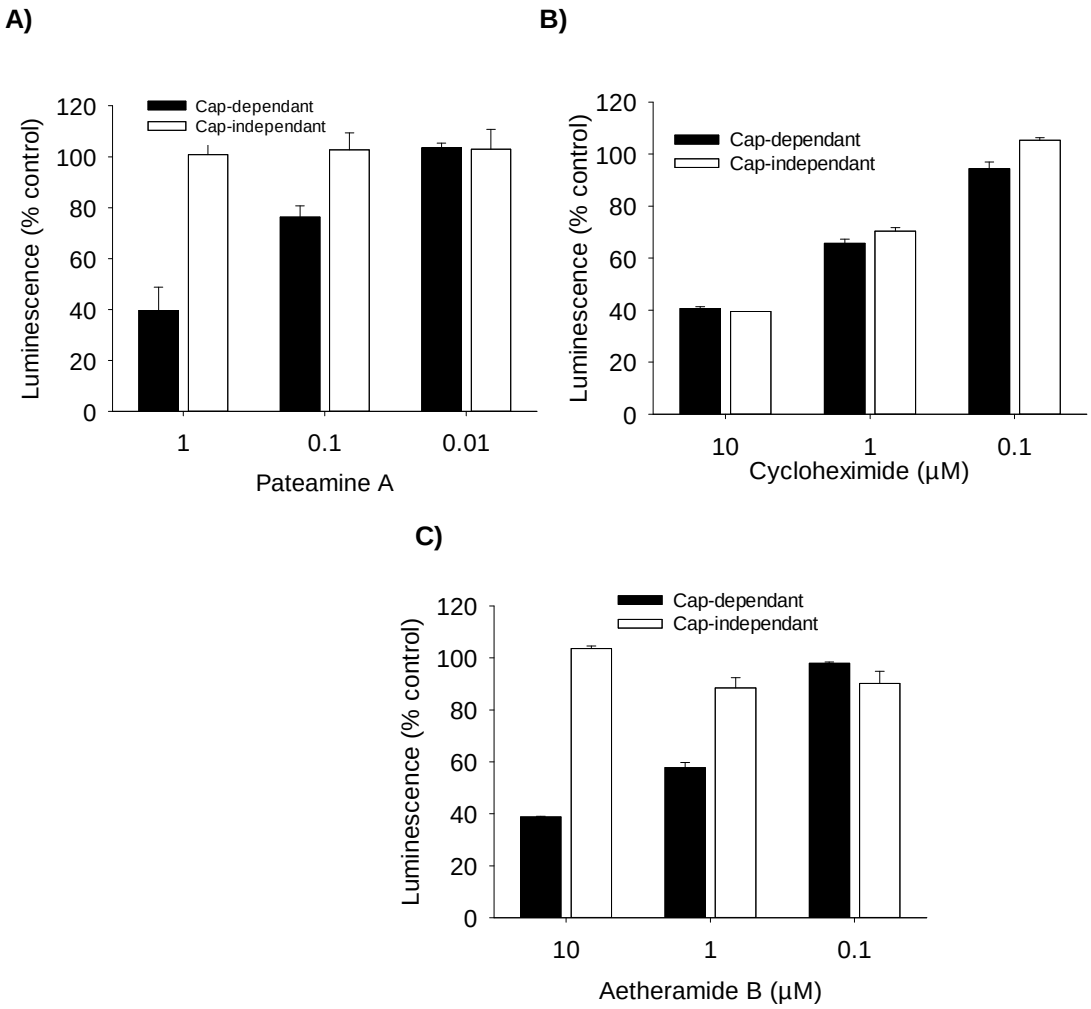



***Figure 3.39: Aetheramide does not inhibit CrPV IRES***

*Aetheramide B (C) like pateamine A (A) inhibited only the cap-dependant translation (black bars) and not the cap-independent translation (white bars) whereas cycloheximide (B) inhibited both the translation systems equally.*

### 3.5.3 Effect of aetheramide B on eIF4 complex

From the previous section it was clear that aetheramide B targets the initiation phase of translation. To test the possibility that aetheramide B could target either eIF4E or eIF4G, the bicistronic polio IRES vector was used. Polio IRES does not require eI4E and eIF4G for translation initiation. Therefore if aetheramide does not inhibit polio IRES dependant translation of Renilla luciferase then it would suggest that aetheramide B targets eIF4 complex. To determine if aetheramide B targets the eIF4 complex, KB-3-1 cells transfected with the polio bicistronic vector was treated with aetheramide B, cycloheximide and pateamine A at concentrations ranging from 10 - 0.01 µM for 3 hours. The luminescence generated from the firefly luciferase representing the cap-dependant translation and Renilla luciferases representing the

cap-independent translation were measured. Cycloheximide, which stalls the elongation phase, and pateamine A, which targets eIF4A, inhibited the translation of Renilla and firefly luciferase reporter systems equally (Fig. 3.40). In contrast to it aetheramide B inhibited the luminescence only from the firefly luciferase suggesting that the target of aetheramide B lies either in eIF4E or eIF4G.

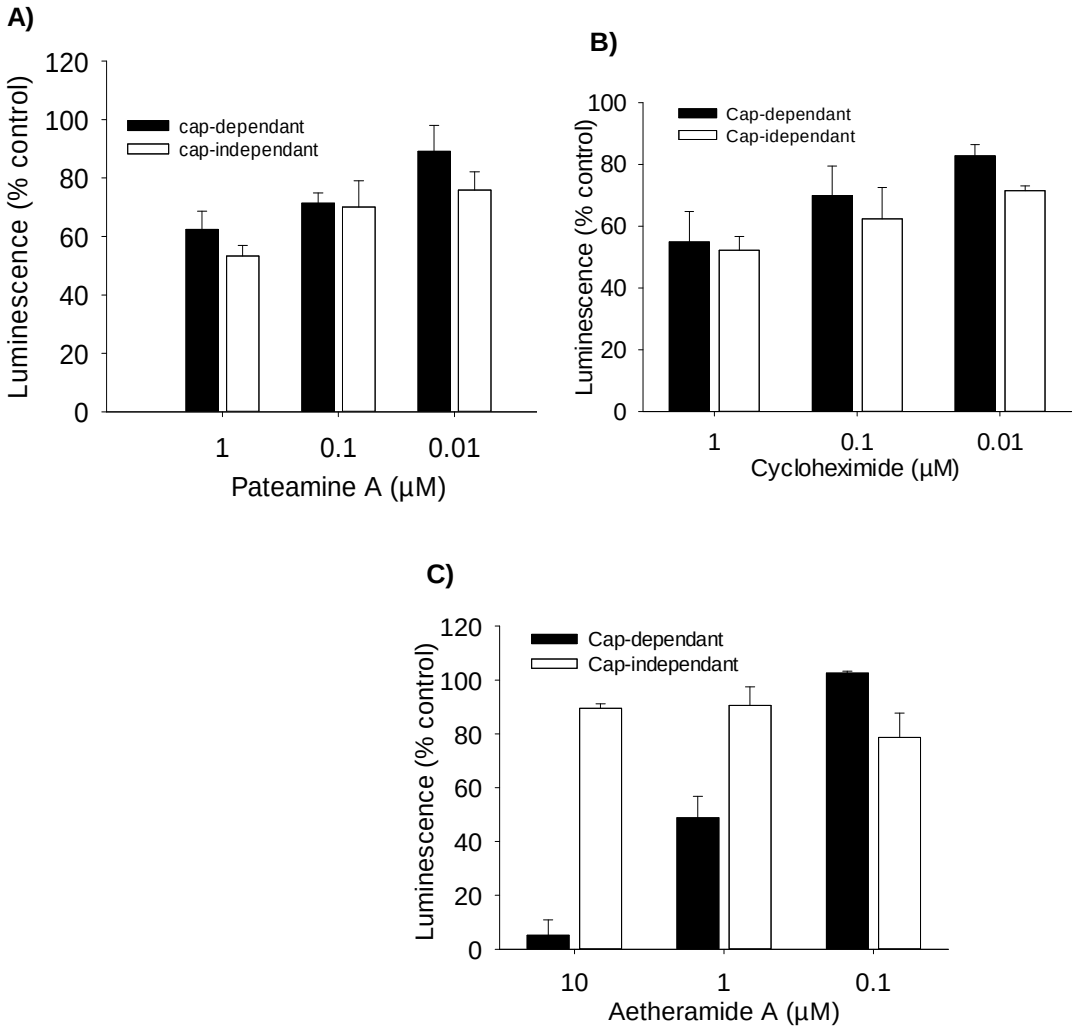



***Figure 3.40: Aetheramide B targets either eIF4E or eIF4G***

*Aetheramide B (C) did not inhibit the cap-independent translation (white bars) but only the cap-dependent translation (white bars). Pateamine A (A) and cycloheximide (B) inhibited both the translation systems.*

### 3.5.4 Mechanism of inhibition of translation initiation by aetheramide B

Aetheramide B does not inhibit the translation of neither CrPV IRES nor polio IRES. This suggests that the possible target of aetheramide B lies either in eIF4E or in eIF4G of the eIF4 complex. As mentioned in the Section 1.2.7, eIF4E can be

regulated by the phosphorylation of 4E-BP. To test if aetheramide B alters the phosphorylation state of 4E-BP, KB-3-1 cells were treated with aetheramide B for 6 hours. The lysed cells were subjected to SDS PAGE followed by western blotting and probed with anti-phosphorylated 4E-BP (Thr 37/46) antibody. Cells treated with methanol served as negative control. Cells treated with LY294002, a known PI3K inhibitor, were used as positive control. As can be seen from Fig. 3.41, aetheramide B induced dephosphorylation of 4E-BP similar to LY294002.

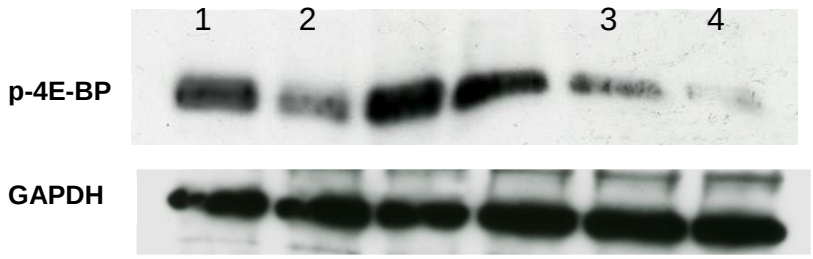



***Figure 3.41: Aetheramide induces dephosphorylation of 4E-BP***

*KB-3-1 cells were treated with either methanol (lane 1), LY294002 (Conc.2.5 µM; lane 2), aetheramide B (1 µM; lane 4) or aetheramide B (100 nM; lane 3) for 6 hours and subjected to western blotting. The blotted membrane was probed with anti-phospho-4E-BP antibody. Cells treated with aetheramide B showed dephosphorylation of 4E-BP like the positive control (lane 2). GAPDH served as a loading control.*

### 3.5.5 Aetheramide B inactivates mTOR

The phosphorylation state of 4E-BP is regulated by the mTOR pathway. 4E-BP is dephosphorylated if mTOR is inhibited. The inhibition of mTOR leads to 4E-BP dephosphorylation (Jackson et al., 2010). To test if mTOR is inhibited by aetheramide, KB-3-1 cells were treated with aetheramide B (1 µM, 100 nM) for 6 hours. Cells treated with 6 µM of LY294002, a known inhibitor of PI3K, were used as a positive control. Cells treated with methanol served as a negative control. The cells were then lysed and subjected to SDS-PAGE followed by western blot. The blotted membrane was then probed with an anti-phospho mTOR (Ser 2448) antibody. As can be seen from Fig. 3.42, cells treated with aetheramide and LY294002 show reduced phosphorylation as compared to cells treated with methanol only.

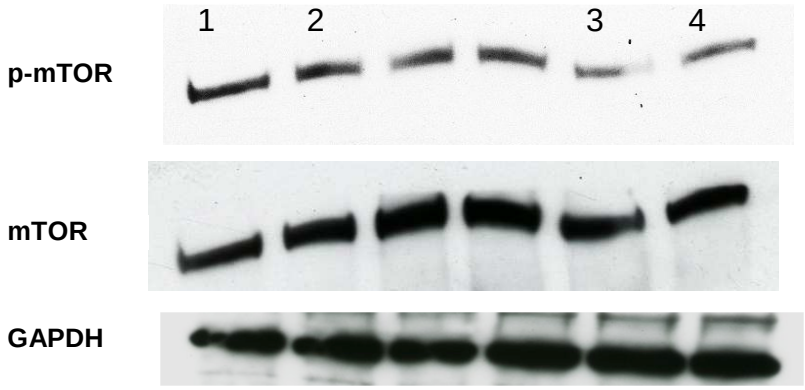



***Figure 3.42: Aetheramide B inhibits mTOR pathway***

*KB-3-1 cells were treated with either methanol (ane 1), LY294002 (con:2.5 µM, lane 2), 1 µM aetheramide B (lane 3) or 100 nM aetheramide B (lane 4) for 6 hours and subjected to western blotting. The blotted membrane was probed with an anti-phospho-mTOR (Ser 2448) antibody, an anti-mTOR and an anti-GAPDH antibody. Cells treated with aetheramide B show reduction in the phosphorylation of mTOR like the positive control (lane 2).The total mTOR protein remained unchanged. GAPDH served as a loading control.*

### 3.5.6 Induction of stress granules by aetheramide B

Aetheramide B induces dephosphorylation of 4E-BP. As a result eIF4E is not available for the formation of an eIF4F complex. This should result in the formation of stress granules. Hence investigating stress granules formation helps to further confirm the target of aetheramide B. To examine if aetheramide B induces stress granules, PtK2 cells were cultured for 16 hours on cover slips in a 4-well plate. The cells were treated with 100 nM of aetheramide B for 4 hours then, fixed and stained for TIA, an RNA binding protein, which served as a marker for stress granules. The cells were also stained for eIF4E. Pateamine A (20 nM) (a known stress granule inducer) was used in positive control cells (Low et al., 2005) (Fig. 3.43). The results showed that aetheramide B indeed induced the formation of stress granules.

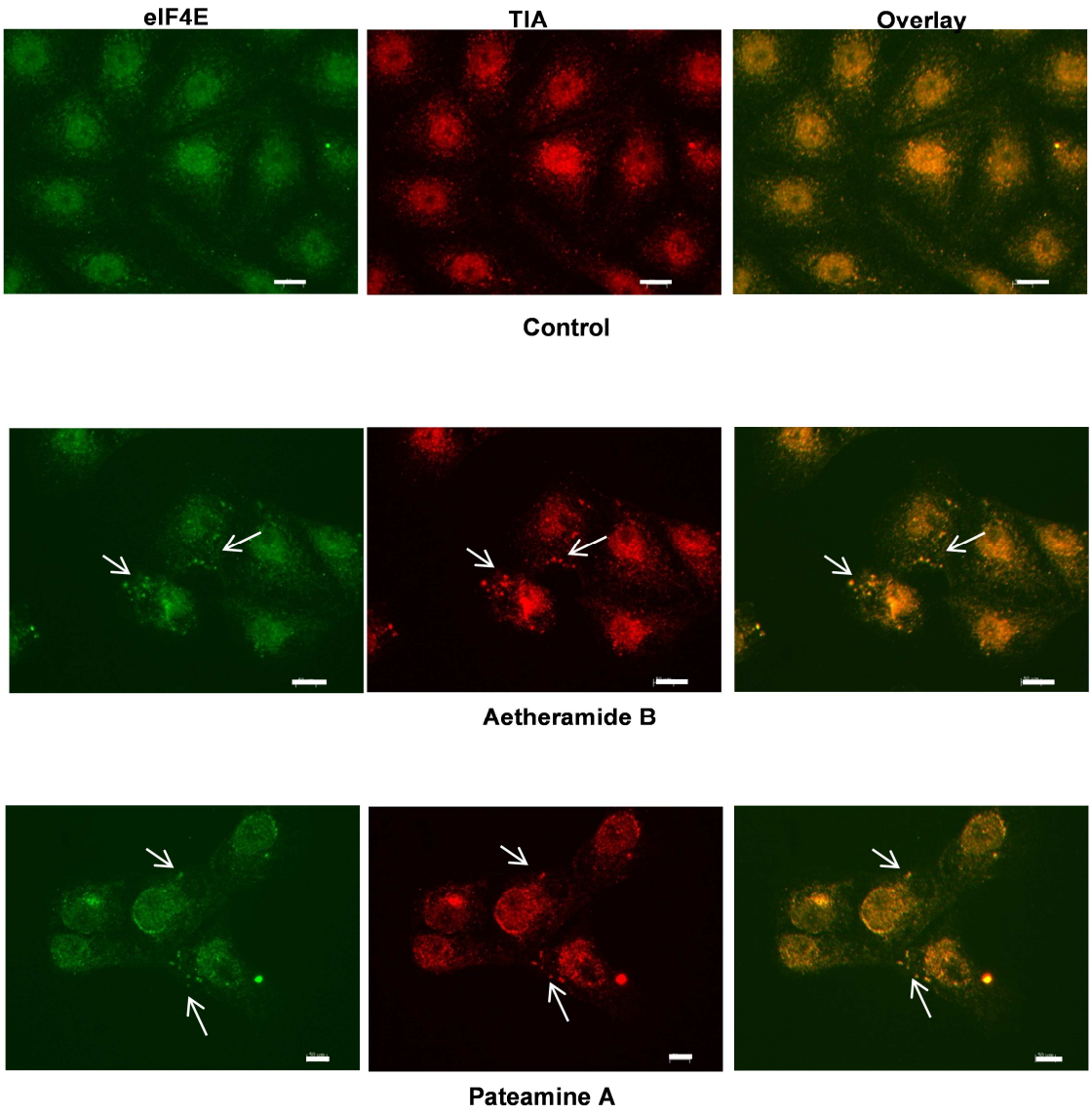



***Figure 3.43: Aetheramide B induces stress granule formation***

*Cultured PtK2 cells were treated with methanol (top row), 100 nM aetheramide B (middle row) or pateamine A (bottom row) for 4 hours. The cells were fixed and stained for eIF4E (green) and TIA (red). Aetheramide B like pateamine A induced stress granules. The arrows indicate stress granules in the cells. Scale bar: 50 µm.*

# 4 Discussion

The molecular mechanism of eukaryotic translation is exceedingly complex and only partially understood as of now. The current knowledge of eukaryotic translation is extrapolation of the knowledge from the prokaryotic system. However, the prokaryotic system varies largely from the eukaryotic translation apparatus. This is clear from the fact that most prokaryotic translation inhibitors are ineffective in the eukaryotic system (Jackson et al., 2010; Poehlsgaard and Douthwaite, 2005; Schroeder et al., 2007; Tu et al., 2005) . Hence there is a dire need for specific eukaryotic translation inhibitors to elucidate the translation machinery. It is clear that protein synthesis is vital for the survival of the cells, but it has also been shown that small molecules targeting the translation system are potential antivirals and anti-tumour agents. Considering the above reasons it is of great importance to find and study the mode of action of translation inhibitors specific to eukaryotes (Chan et al., 2004). Eukaryotic translation inhibitors can stall translation by three different mechanisms: i) by depleting the substrates that are required for the translation, ii) by directly or indirectly targeting the factors involved in translation, and iii) by binding to the ribosome directly and inactivating them (Sonenberg and Hinnebusch, 2009).

The central objective of this study was to elucidate the mode of action of four small molecules that are translation inhibitors of the eukaryotic systems viz. gephyronic acid, myriaporone 3/4, des-epoxy tedanolide and aetheramide B. The first three polyketides have been reported as translation inhibitors earlier whereas aetheramide B was identified as a translation inhibitor in the course of this study. In the following the results of the four polyketide translation inhibitors are discussed.

## 4.1 Polyketides inhibit eukaryotic translation

Gephyronic acid, myriaporone 3/4, des-epoxy tedanolide and aetheramide B inhibited the growth of cancer cells in the nanomolar ranges. They also inhibited the growth of eukaryotic micro-organisms like *S. cerevisiae and C. albicans* as reported earlier (Hines et al., 2006; Plaza et al., 2012; Sasse et al., 1995; Taylor, 2008). In line with the earlier reports gephyronic acid, myriaporone 3/4 and des-epoxy tedanolide did not inhibit the growth of any of the prokaryotes used in this study. Gephyronic acid, des-epoxy tedanolide and myriaporone 3/4 were already established as translation inhibitors. Consistent with the previously published results, the three polyketides proved to be translation inhibitors with an $IC_{50}$ of 0.07 µM (gephyronic acid), 0.4 µM

(myriaporone 3/4), 0.01 µM (des-epoxy tedanolide) in the *in vitro* rabbit reticulocyte translation system and 30 nM (gephyronic acid), 100 nM (myriaporone 3/4), and 0.2 nM (des-epoxy tedanolide) in KB-3-1 cells. Aetheramide B is a novel translation inhibitor reported for the first time in this study. It inhibited translation with an $IC_{50}$ of 1.1 µM in the *in vitro* translation and 700 nM in KB-3-1 cells.

## 4.2 Mechanism of translation inhibition by the polyketides

### 4.2.1 Gephyronic acid

It has been shown by a number of studies that induction of apoptosis by various stimuli could lead to inhibition of protein synthesis (Clemens et al., 2000). Therefore A-431 cells were treated with gephyronic acid and analysed for apoptosis at various time points. However, from the results (Fig. 3.3) it is clear that after 6 hours of treatment with 50 nM gephyronic acid only 16.2% cells had undergone apoptosis whereas at the same concentration after 4 hours there was a translation inhibition of more than 40% (Fig. 3.2) This indicated that translation inhibition precedes apoptosis. The cells underwent apoptosis as a result of translation inhibition. Also the correlation between the $IC_{50}$ of cellular cytotoxicity and cellular translation holds proof that gephyronic acid is indeed a translation inhibitor as reported previously (Sasse et al., 1995). The cytotoxicity of gephyronic acid was partially reversible at lower concentrations and only when incubated not longer than 6 hours. Incubation at higher concentration or for longer time periods with gephyronic acid induced an irreversible cytotoxicity. This suggests that gephyronic acid induces apoptosis only as result of translation inhibition unlike compounds like pateamine A. In case of pateamine A caspase 3 induced cleavage of translation factors was reported to be an additional reason for translation inhibition (Hood et al., 2001).

Gephyronic acid inhibited the translation of the cap-dependant IRES. However, it did not inhibit the translation of Renilla luciferase encoded by the CrPV IRES (Fig. 3.9). As reported by others, pateamine A which targets the initiation phase inhibited only cap-dependant translation. Cycloheximide, on the other hand inhibited the translation of both cap-dependent and cap-independent translation equally as reported earlier (Schneider-Poetsch et al., 2010). This confirmed that the target of gephyronic acid lies within the initiation phase as CrPV IRES does not require any of the initiation factors. Also, gephyronic acid inhibited the translation of the Renilla luciferase encoded by the polio IRES (Fig. 3.10). This eliminated the possibility that target of gephyronic acid could lie with either eIF4E or eIF4G. From the chemo genetic interaction screening,

the target was further shortlisted to the eIF2 complex. With the help of a DARTS approach, the direct target of gephyronic acid was identified to be eIF2α. Pull down assays and immunofluorescence studies confirmed that gephyronic acid binds directly to eIF2α. The affinity of gephyronic acid for eIF2α is strong as can been seen from the western blots of the pull down assay. Previously, compounds like salubrinal and #1181 have been shown to have an effect on eIF2α (Boyce et al., 2005; Natarajan et al., 2004). Salubrinal inhibited de-phosphorylation of eIF2α whereas #1181, induced phosphorylation by activating PERK. PERK is an eIF2α kinase which is activated when $Ca^{2+}$ is depleted (Harding et al., 1999). In both cases the effect of the compounds on eIF2α is only indirect effect. So gephyronic acid is the first small molecule that binds to eIF2α directly.

It was shown that gephyronic acid is 100 fold less active in primary cells (NHDF) when compared to the cancerous KB-3-1 cells. This may not be due to the differences in the sensitivities of different cell lines to apoptosis rather due to alterations in the translation apparatus. Some cancers like colon cancer have shown to overexpress eIF2α (Rosenwald et al., 2003). There could be an inherent alteration in eIF2α of cancer cells making them more sensitive to treatment with gephyronic acid. It could also be possible that gephyronic acid does not target eIF2α of primary cell lines. This was reported in case of candidaspongiolide which inhibits translation by phosphorylation of eIF2α. However, candidaspongiolide did not induce phosphorylation of eIF2α in primary fibroblast cells (Trisciuoglio et al., 2008). This could also be possible in the case of gephyronic acid.

Recent studies showed that inhibition of translation can inhibit angiogenesis in tumours (Cencic et al., 2009; Graff et al., 2007). Here, the results show that gephyronic acid inhibited the tube formation by HUVECs. This is in correlation with previously published data with translation inhibitors like Didemnin B and cytotrienin A (Lindqvist et al., 2010). Further studies are required to show if gephyronic acid can inhibit angiogenesis *in vivo.*

In line with previous publication by Sasse et al., (1995), gephyronic acid was shown to be cytotoxic only in eukaryotes and not in prokaryotes. Translation inhibition assays with *E. coli* S30 extracts shows that gephyronic acid failed to inhibit translation of the luciferase mRNA, whereas, gephyronic acid inhibited translation of luciferase mRNA in both wheat germ extract and in rabbit reticulocyte lysate extract. This shows that gephyronic acid is a specific eukaryotic translation inhibitor. Gephyronic acid could not inhibit the translation in the *E. coli* system which explains its inactivity in prokaryotes.

The reason that gephyronic acid did not inhibit the prokaryotic translation could be because its target eIF2α is conserved in eukaryotes and differs greatly from prokaryotic initiation factor 2 (Ito et al., 2004). An alignment of the amino acid sequence using ClustalW2 reveals that the amino acid sequence between position 6 and 120 are highly conserved in eukaryotes (Fig 4.1). On the other hand, there is no sequence similarity between prokaryotes and eukaryotes.

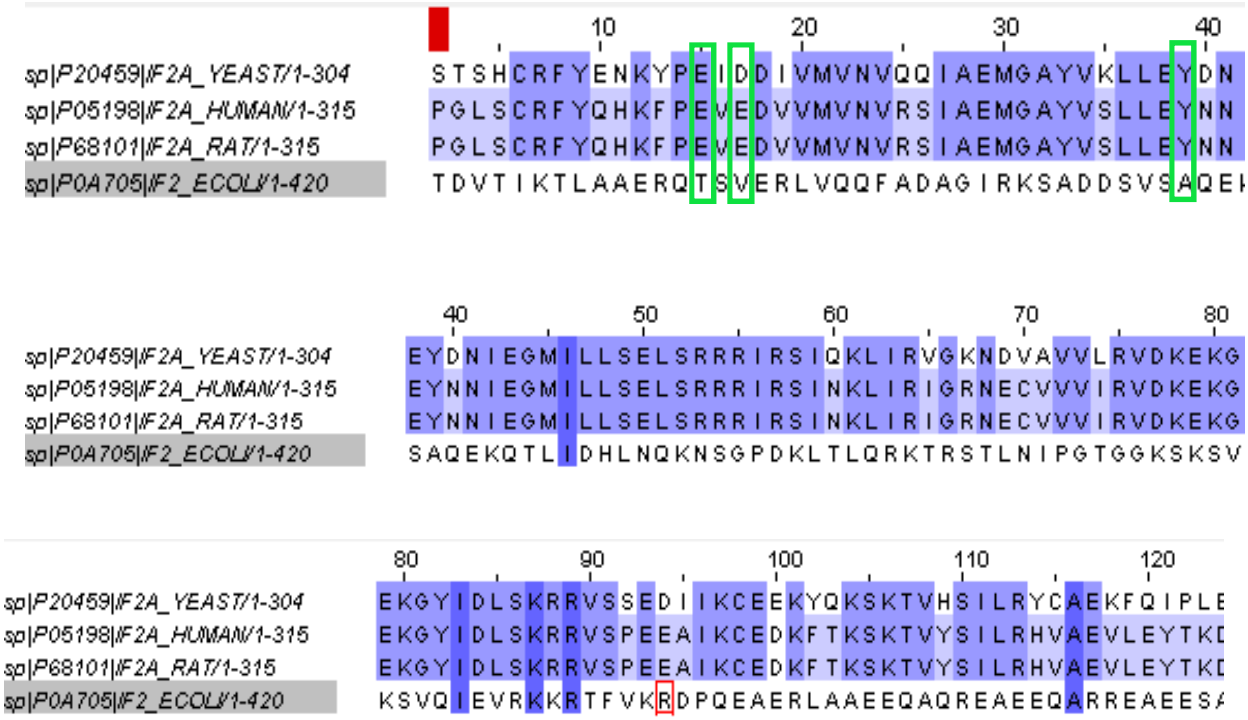



***Figure 4.1: Sequence alignment of eIF2α***

*Protein sequence alignment of eIF2α from yeast, humans, rat and E. coli shows that amino acids at position 15, position 17 and position 39 (marked in green boxes) are different in eukaryotes and prokaryotes. The protein sequences for the alignment were obtained from UniProt.*

Also the amino acids which gephyronic acid binds to (Glu 15, Tyr 38) are different in eukaryotes and prokaryotes. Tyr 38 to which gephyronic acid strongly binds is conserved only among eukaryotic species. The other residues like Glu 14 and Asp 17 present in eukaryotes are replaced by Thr 15 and Val 17 in *E. coli*. The differences in these residues possibly alter the binding affinity of gephyronic acid for eIF2α. As a result gephyronic acid does not have a target in the prokaryotic system making them least susceptible to gephyronic acid.

### 4.2.2 Myriaporone 3/4

Myriaporone 3/4 was reported as a translation inhibitor (Hines et al., 2006). However their publication did not report the mechanism by which myriaporone 3/4 inhibits translation. During the course of this study, synthetic myriaporone 3/4 was used to elucidate the mode of action of the polyketide, which was was obtained from Prof. Rich Taylor, (Notre Dame, USA). As reported earlier myriaporone 3/4 proved to be cytotoxic in nanomolar ranges and also inhibited translation with a similar $IC_{50}$. Results from the apoptosis study showed that myriaporone 3/4 (90 nM) induced 11 % cell death after 3 hours compared to 5 % cell death in the control. However, after 3 hours of treatment with myriaporone 3/4 (90 nM), the translation was inhibited by 50 % (in KB-3-1 cells). This clearly shows that apoptosis happens as a result of translation inhibition and not vice versa. This is also in line with the previous published results where myriaporone 3/4 caused delay in the S phase and then induced apoptosis (Hines et al., 2006).

Myriaporone 3/4 inhibited the translation of Renilla luciferase translated by both polio and CrPV IRES similar to cycloheximide. This shows that myriaporone 3/4 does not target the initiation phase and the target possibly lies in the elongation and termination phase. Using a chemo genetic interaction screening the target was placed within the elongation phase (Table 3.4). Since the elongation factor 1 mutant did not show either increased or descried sensitivity compared to the wild type, elongation phase 2 was studied further. Though yeast has another elongation factor, eEF3 (Kamath and Chakraburtty, 1989), the mutant was not a viable one and hence could not be used in the screening. The well-established regulation mechanism within the elongation phase other than eEF1A inhibition is induction of eEF2 phosphorylation. When cells treated with myriaporone 3/4 were tested for the phosphorylation state of eEF2, they showed an increase in the phosphorylation of eEF2 (Fig. 3.26 and 3.27). The only kinase known to phosphorylate eEF2 was eEF2K. Therefore the phosphorylation state of eEF2K was analysed. However, the phosphorylation of eEF2K was not altered by the treatment with myriaporone 3/4. It was then hypothesised that myriaporone 3/4 binds directly to eEF2K thereby activating it. The results from a DARTS approach (Fig. 3.29) and a translation inhibition assay supplemented with enriched eEF2K lysate (Fig. 3.30) showed that indeed myriaporone 3/4 binds directly to eEF2K.

There has been previous reports of small molecules NH125 (synthetic) (Arora et al., 2003), TS2 (Cho et al., 2000) and rottlerin isolated from *Mallotus phillippinensis* as inhibitors of eEF2K (Gschwendt et al., 1994). The potency of the above mentioned

compounds were in micromolar ranges. Myriaporone 3/4 on the other hand was potent in the nanomolar ranges making it the most potent eEF2K inhibitor. Rottlerin inhibits eEF2K by affecting the $Ca^{2+}$ channels and inhibiting the protein kinase C (PKC) pathway (Gschwendt et al., 1994). Inactivation of PKC leads to the phosphorylation of eEF2 via eEF2K (Devost et al., 2008). Therefore the phosphorylation of eEF2 induced by rottlerin is only a secondary effect. NH125 was initially reported as direct inhibitor of eEF2K. This was later proved incorrect as NH125 induces phosphorylation of eEF2 via AMPK and competing with calmodulin for eEF2K (Chen et al., 2011). TS2 a synthetic small molecule inhibited by binding to eEF2K. TS2 binds to the ATP binding site (Cho et al., 2000). Thus, myriaporone 3/4 is the first natural compound to inhibit translation via direct interaction with eEF2K.

Myriaporone 3/4 showed activity only in eukaryotes and did not have any cytotoxic effects in prokaryotes used in the screening (Table 3.2). This was also reported in earlier studies (Hines et al., 2006). This is probably due to the fact that prokaryotes do not have eEF2K. As myriaporone 3/4 does not have a target in the prokaryotic system it cannot inhibit translation of the prokaryotic system.

Myriporone 3/4 showed at least 300 fold less cytotoxicity in primary cells when compared to cancerous KB-3-1 cells even after 72 hours treatment. This could be due better uptake of myriaporone 3/4 by cancer cells due to their increased metabolism. It has also been shown that phosphorylation of eEF2 can lead to ER stress (Boyce et al., 2008). As the cancer cells are already under stressed conditions even a slight increase could be deleterious to their survival. As a result the ER stress could probably lead to increased apoptosis. Further investigations are still required to assess the difference in cytotoxicity induced by myriaporone 3/4 in cancer and primary cells. Also if this difference could be extrapolated to in vivo models have to be studied.

Myriaporone 3/4 inhibits angiogenesis as shown by HUVECs in the tube formation assay in nanomolar ranges. Cytotrienin A, a previously reported translation elongation inhibitor inhibited tube formation in micromolar ranges (Lindqvist et al., 2010). Thus myriaporone 3/4 proves to be a more potent angiogenesis inhibitor. Though myriaporone 3/4 is less toxic to primary cells it inhibits the formation of vessels by HUVECs on Matrigel®. This is plausible as it has been shown that induction of phosphorylation of eEF2 inhibits migration of endothelial cells (Khan et al., 2010). Though myriaporone 3/4 does not show cytotoxicity to a greater extent in primary

cells, it inhibits the migration of HUVECs which subsequently lead to the inhibition of angiogenesis.

eEF2K is overexpressed in most cancers (Chen et al., 2011). As a result binding of myriaporone 3/4 to eEF2K could activate it thus inducing phosphorylation of eEF2 and finally inhibiting translation. eEF2K is also involved in the switch between apoptosis and autophagy (Cheng et al., 2012; Hait et al., 2006). Inhibiting eEF2K would inhibit the signalling required for autophagy and thus lead to cell death. Cheng et al. (2012), reported that inhibiting eEF2K causes the tumour cells undergo apoptosis rather than switch to autophagy. Considering the above statements it is clear that myriaporone 3/4 has a potential to be used as a potent anti-cancer agent.

### 4.2.3 Des-epoxy tedanolide

Des-epoxy tedanolide is a synthetic analogue of 13-desoxy tedanolide (Diaz et al., 2012b). The only difference is the lack of epoxide at C17. It has been shown previously that the conformation is controlled by the configuration at C15 and C5. It has been proposed that the removal of the epoxide decreases the activity of the compound by at least a hundred fold (Hines et al., 2006; Taylor, 2008). However, des-epoxy tedanolide shows only one fold difference (reduction) in cytotoxicity when compared to 13-deoxy tedanolide (Diaz et al., 2012b). Thus the epoxide may not be as important for its activity as proposed earlier. Also the epoxide was predicted to be necessary for the formation of covalent bonds (Hines et al., 2006; Taylor, 2008). Thus the role of the epoxide in 13-desoxytedanolide requires further investigations.

The des-epoxy tedanolide was provided by Prof. Markus Kalesse (Hannover, Germany). Des-epoxy tedanolide was active only in eukaryotes and did not inhibit the growth of prokaryotes. This also draws a parallel with the published result that des-epoxy tedanolide inhibits only the eukaryotic translation and not the prokaryotic translation. It has been shown that 13-desoxy tedanolide binds to the 60S ribosome and induces ribotoxic stress by binding to 60S ribosome (Schroeder et al., 2007). However, it has been suggested that ribotoxic stress cannot induce cell death with such great potency as 13-desoxtedanolide does. Therefore another mechanism must be responsible for the potency of 13-desoxtedanolide in addition to induction of ribotoxic stress. Candidaspongiolide, a member of the tedanolide family (Fig. 1.15) showed that it induced cell death by stalling translation. Candidaspongiolide inhibited translation by inducing phosphorylation of eIF2α (Meragelman et al., 2007). When des-epoxy tedanolide treated cells were tested for the phosphorylation of eIF2α, the

cells showed an increased level of eIF2α phosphorylation. However, the mode of phosphorylation of eIF2α is yet to be identified. eIF2α can be phosphorylated by 4 different kinases viz. GCN2, PKR, PERK and HRK (Jackson et al., 2010). So further studies need to be carried out to find the kinase involved in the phosphorylation of eIF2α. The increased phosphorylation of eIF2α in cells when treated with des-epoxy tedanolide could also because of the inhibition of GADD34, an eIF2α dephosphorylase as seen in the case of salubirinal (Boyce et al., 2005). This is yet to be investigated.

A bicistronic vector was used to locate the translation phase that des-epoxy tedanolide targets. Des-epoxy tedanolide inhibited the translation of both CrPV and polio IRES dependent translation (Fig. 3.34, 3.35). As des-epoxy tedanolide induces phosphorylation of eIF2α it was expected that it would inhibit polio IRES dependant translation. However, it also inhibited the translation of CrPV IRES which does not require any of the initiation factors. Therefore it was proposed that there should be another target either in the elongation or in the termination phase of translation.

The yeast mutant collection could not be used for chemo genetics studies as des-epoxy tedanolide did not inhibit the growth of *S.cerivesiae*. Therefore the phosphorylation state of eEF2 was analysed. It was seen that des-epoxy induced phosphorylation of eEF2 though the eEF2K. It has been reported recently that salubrinal, an eIF2α phosphorylation inducer, induces phosphorylation of eEF2 via the AMPK pathway. Phosphorylation of eIF2α can lead to ER stress which in turn leads to eEF2 phosphorylation (Boyce et al., 2008). Therefore the induction of eEF2 phosphorylation may not be an direct effect of des-epoxy tedanolide.

Des-epoxy tedanolide also inhibited the tube formation of HUVECs plated on Matrigel®. The inhibition of translation could be because of two reasons. Firstly, des-epoxy tedanolide is a potent cytotoxic compound with an $IC_{50}$ of 1.6 nM. As result of this it could be inducing apoptosis in HUVECs which in turn inhibits translation. Secondly, as mentioned earlier phosphorylation of eEF2 inhibits the migration of HUVECs which in turn inhibits tube formation.

Des-epoxy tedanolide induces apoptosis in 33 % cells compared to 5 % in the control cells within six hours. This is significantly higher when compared to the two previous mentioned compounds viz. gephyronic acid and myriaporone 3/4. There are two plausible explanations for this. Firstly, des-epoxy tedanolide being more potent induces higher cell death rates. It has also been reported that the cytotoxicity induced

by it is irreversible even at lower concentrations which suggest that des-epoxy tedanolide could be forming a covalent bond with its target (Diaz et al., 2012b). As a result it has a significant higher potency. Secondly, 13-desoxytedanolide has been shown to induce ribotoxic stress (Lee et al., 2006). Des-epoxy tedanolide like 13-desoxy tedanolide could induce stress granules in addition to the induction of phosphorylation of eIF2α and eEF2. Phosphorylation of eEF2 has shown to induce ER stress. The combination of ribotoxic stress, ER stress and translation inhibition could result in a greater potency of des-epoxy tedanolide. However, the induction of ribotoxic stress by des-epoxy tedanolide was not studied during the course of this study. It would be interesting to check if des-epoxy induces ribotoxic stress.

### 4.2.4 Aetheramide B

Aetheramide B is different from the other polyketides in this study as it has two amino acids within its structure. Plaza et al. (2010) reported the isolation of aetheramide A and B from myxobacteria. Though two isomers of aetheramide were reported, only aetheramide B was used for this study. The report did not mention anything about its mode of action. During the course of this study it was identified as a translation inhibitor.

The cytotoxicity of aetheramide B in various cell lines was in the nanomolar range with an $IC_{50}$ values between 15 to 40 nM. It was reported that aetheramide showed cytotoxicity with an $IC_{50}$ of 110 nM in colon cancer cell lines. The results from this study show that aetheramide B is slightly more potent. This lower activity could be because the colon cells are more resistant to aetheramide B compared to the other cell lines used during the course of this study.

As a routine screening in our laboratory suggested that aetheramide B could be a translation inhibitor, translation inhibition assays were performed with rabbit reticulocyte cell lysate and with KB-3-1 cells. Aetheramide B inhibited translation in KB-3-1 cells with an $IC_{50}$ of 700 nM. To assess the phase of the translation targeted by aetheramide B, bicistronic vectors with either polio IRES or CrPV were used. Aetheramide B inhibited the translation of cap-dependant translation in both the vector transfected cells. However, it did not inhibit both the IRES dependant translation. This suggested that the target of aetheramide B lays within the initiation phase and either targets eIF4E or eIF4G.

If there is a disruption of the eIF4 complex, formation of stress granules is seen (Buchan and Parker, 2009). When cells treated with aetheramide B were tested for stress granules, they indeed showed the presence of stress granules. This confirmed that the eIF4 complex is targeted, either directly or indirectly. eIF4E is regulated by altering the phosphorylation of 4E-BP (Morino et al., 2000). Therefore the phosphorylation state of 4E-BP was tested. Dephosphorylation of 4E-BP was observed. The phosphorylation state of 4E-BP is regulated by mTOR. If mTOR is deactivated then 4E-BP is dephosphorylated (Jackson et al., 2010). Cells treated with aetheramide B showed reduced phosphorylation of mTOR suggesting that aetheramide B targets the mTOR pathway.

There have earlier been reports on mTOR inhibitors. Rapamycin is a well-studied mTOR inhibitor. However, rapamycin is not an mTOR specific inhibitor. It has also been shown to inhibit phospho inositol-3-kinase (PI3K). Most of the other mTOR inhibitors reported are derivatives of rapamycin (rapalogs), for example temsirolimus (Del Bufalo et al., 2006). The current results with aetheramide B suggest that it deactivates mTOR pathway. However, it is unclear if the deactivation of mTOR by aetheramide is because it targets mTOR or if aetheramide has a target upstream of mTOR. Therefore the pathway upstream of the mTOR signalling has yet to be studied.

Aetheramide B was also shown to inhibit angiogenesis in this study with an $IC_{50}$ of 40 nM. It was shown earlier that rapamycin could inhibit angiogenesis (Del Bufalo et al., 2006). It has been reported by a number of researchers that PI3K/AKT/mTOR signalling is very critical for angiogenesis (Jazirehi et al., 2012; Nicoletti et al., 2011; Sheppard et al., 2012). Thus inhibiting the pI3K/AKT/mTOR pathway has a profound impact on HUVECs to form vessels both *in vivo* and *in vitro*. This explains the potent effect of aetheramide B in inhibiting the tube formation by HUVECs plated on Matrigel®.

Aetheramide B inhibits HIV with an $IC_{50}$ of 15 nM (Plaza et al., 2012). Recently it has been shown that during HIV infection the mTOR signalling pathway is up-regulated and leads to a hyper-phosphorylation of 4E-BP. This then led to the increase in the translation of proteins in HIV infected cells (Rehman et al., 2012). It was also reported that rapamycin possesses anti-HIV properties (Donia et al., 2010). This explains the inhibition of HIV proliferation by aetheramide B. The $IC_{50}$ of translation inhibition by aetheramide was 0.7 µM, but aetheramide B inhibited HIV proliferation with an $IC_{50}$ of 15 nM. Thus the effect of aetheramide B on HIV proliferation is significantly higher

than its effect on translation inhibition. This could be because mTOR activation is very vital for HIV replication and even a slight deactivation of the mTOR signalling has a great impact on its replication (Nicoletti et al., 2011; Rehman et al., 2012). It could also be possible that aetheramide B has another target in the cell which is essential for HIV infection and replication. Thus, inhibiting multiple pathways required for HIV propagation, aetheramide B has a more profound effect on its proliferation and infection.

## 4.3 Structurally similar but different targets

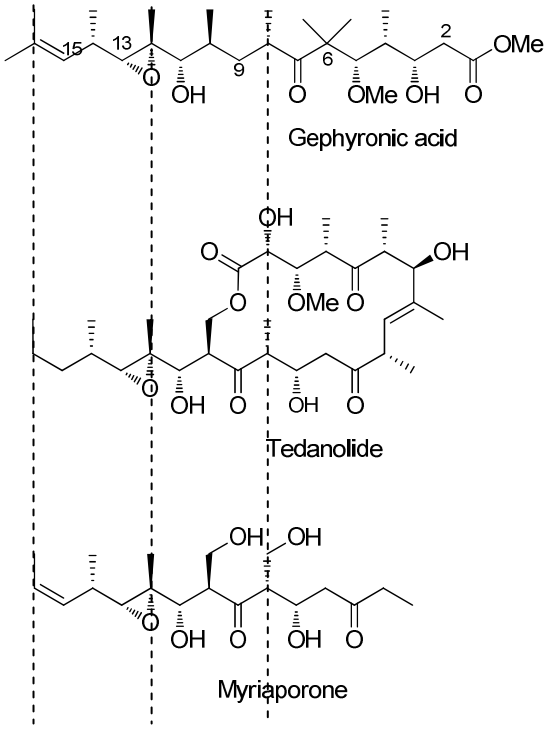



***Figure 4.2: Structural relationship between the polyketides***

*The "southern hemisphere" of the three polyketides resembles each other.*

Three out of the four polyketides in this study are structurally similar viz. gephyronic acid, myriaporone 3/4 and tedanolide at the "southern hemisphere" (Taylor, 2008). As the three polyketides have structural similarities and the southern hemisphere was important for their function, it was proposed that they could have similar targets (Diaz et al., 2012a). Though myriaporone 3/4 and des-epoxy tedanolide induce phosphorylation of eEF2 it is through different mechanisms. Gephyronic acid on the other hand targets translation by binding to eIF2α. From the current results it is clear that these polyketides target different proteins or pathways irrespective of their structural similarity.

# 5 Outlook

Our current understanding of the eukaryotic translation system is only half-done. The prokaryotic system is well characterised due to the availability of wide range of prokaryotic translation inhibitors. Identifying new eukaryotic inhibitors and elucidating their modes thus plays a critical role. This study identified four translation inhibitors that are specific to eukaryotes.

All the four compounds in this study have shown to be specific eukaryotic inhibitors. And hence they can be used to study the eukaryotic translation better. Gephyronic acid inhibited translation by binding to eIF2α. This is the first compound that has been reported to do so. To understand the structural features of eIF2α better, a co-crystallisation of gephyronic acid with eIF2α will be extremely valuable. Gephyronic acid can also help in understanding the role of eIF2α in translation initiation better. Myriaporone 3/4 is the first natural compound to target eEF2K directly. eEF2K is only partially understood and it is interesting to note that bacteria and yeast lack eEF2K. With the help of myriaporone 3/4 crystallisation of eEF2K which was not possible until now might be possible. This would give new insights into the regulation of the elongation phase. Aetheramide B inhibits the mTOR pathway. Further studies have to be carried out to test if it is a specific mTOR inhibitor. Aetheramide B was shown to inhibit HIV *in vitro*. However, there was a difference in its $IC_{50}$ values of translation inhibition and viral inhibition. This suggests that there is a possible second target for aetheramide B.

The four small molecules in this study were shown to have anti-cancer properties. They are cytotoxic to transformed cells, inhibit angiogenesis and hence they should be tested *in vivo* tumour models for their efficacy as a possible anti-cancer agent. Myriaporone 3/4 and gephyronic acid can be used to elucidate the role of eEF2K and eIF2α, in tumour progression.

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# Curriculum Vitae

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2000-2002 Pre-University Education

## Academic Background

2002-2005 Bachelor of Science in Biochemistry, Bharathiar University, India

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## Publications

Muthukumar, Y., Roy, M., Raja, A., Taylor, R., Sasse, F*. The marine polyketide myriaporone 3/4 stalls translation by targeting the elongation phase. ChemBioChem. In press. DOI: 10.1002/cbic.201200522 (2012).

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